Exploring proteomic and microbiome profiling in pigs fed high fibre diets

by

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Declaration

I, Arnold Tapera Kanengoni, declare that this dissertation which I have compiled and submitted to the Stellenbosch University for the Doctoral degree, represents my own work and has never been submitted to any other tertiary institution for any degree.

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Abstract

The aim of this study was to explore proteomics and microbiome profiling in pigs fed high fibre diets. In the first phase, maize cobs were ensiled using whey, molasses and exogenous enzymes in the laboratory and effects on ensiling characteristics and fibre levels were evaluated. In the second phase South African Windsnyer-type Indigenous pigs (SAWIP) and Large White x Landrace crosses (LW x LR) were fed diets containing ensiled maize cobs and evaluated on; diet preferences, nutrient digestibility and colonic fermentation; growth performance, carcass traits and blood metabolite profiles; the faecal microbiome; and serum and liver proteomic profiles. Ensiling maize cobs with molasses, whey and exogenous enzymes did not improve fermentation characteristics but exogenous enzymes reduced fibre fractions and energy content of maize cob silages. Dets containing two levels of maize cobs ensiled without any additive; a low (LMC) and high (HMC) maize cob inclusion levels and a control diet which did not have any maize cobs (CON) were formulated. The SAWIP preferred the CON diet more than ($P < 0.05$) diets with maize cobs while the LW x LR had no feed preferences. There was no correlation between preference and diet digestibility in both breeds. The SAWIP digested nutrients better ($P < 0.05$) than the LW x LR in the high fibre diets. There were no differences in the diversity of the core composition of gut bacterial communities between the breeds and diets. There were differences in the ratios of *Bacteroidia* to *Clostridia* between the SAWIP and LW x LR. *Verrucomicrobiae* was present in SAWIP and LW x LR on HMC diet and not on the CON diet. There was a breed x diet interaction ($P < 0.05$) for *Oscillospira*. Analysis of the microbiome revealed breed differences and no dietary differences. There were differences in serum and liver proteins and in serum metabolite levels. Two specific proteins identified were *Guanidinoacetate N-methyltransferase-like* isoform 1 associated with creatine biosynthetic and *Catalase*, which is involved in cholesterol metabolic processes. At the grower stage, the SAWIP consumed more feed per metabolic body weight than the LW x LR while at the finisher stage LW x LR consumed more feed per metabolic body weight ($P < 0.05$) than the SAWIP. The breed of pig influenced most of the growth performance and carcass parameters more than the diet did. The SAWIP demonstrated an adaptation to high fibre diets by consuming more feed than the LW x LR per metabolic body weight at the grower stage. The inclusion of ensiled maize cobs in diets did not negatively affect selected commercial pork cuts. Analysis of faecal microbiomes revealed differences that may explain the enhanced ability of the SAWIP to digest fibrous diets better than the LW x LR breed.Proteomics can identify biomarkers that evaluate the performance of pigs consuming high fibre diets. A proof of principle to assess serum and liver protein profiles of pigs fed a a high fibre diet using a sodium dodecyl sulphate polyacrylamide gel electrophoresis matrix-assisted laser desorption ionization mass spectrometry (SDS-PAGE /MALDI MS) workflow was established.

Key words: ensiling, exogenous enzymes, palatability, fermentation, fibre, metageome, biomarkers

Opsomming

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List of Abbreviations

AA	acetic acid
ADF	acid detergent fibre
ADL	acid detergent lignin
αNDF	α-amylase neutral detergent fibre
ANF	anti-nutritional factor
BA	butyric acid
BC	buffering capacity
CLA	conjugated linoleic acid
CON	control
CP	crude protein
DDGS	distillers dried grains with solubles
DE	digestible energy
DF	dietary fibre
DGGE	denaturant gradient gel electrophoresis
DM	dry matter
DNA	deoxyribonucleic acid
EE	ether extract
ESI-MS	electrospray ionization MS
GC	gas chromatography
GE	gross energy
GIP	glucose dependent insulinotropic peptide

HPLC	high-pressure liquid chromatography
LA	lactic acid
LAB	lactic acid bacteria
LC	liquid chromatography
LW	Large White
MALDI-TOF-MS	Matrix-assisted laser desorption ionization with a time-of-flight MS
MS	mass spectrometry
NDF	neutral detergent fibre
NH_3-N	ammonia nitrogen
SAS	Statistical Analysis Systems
SAWIP	South African Windsnyer-type Indigenous pigs
SCFA	short chain fatty acids
SD	standard deviation
SELDI-TOF-MS	surface-enhanced laser desorption ionization with a time-of-flight MS
TI	trypsin inhibitor
TIA	trypsin inhibitor activity
v/v	volume/volume
VCP	valosin-containing protein
VFA	volatile fatty acids
WSC	water-soluble carbohydrates
ZnO	zinc oxide

Chapter 1

1 Introduction

1.1 General Introduction

Pig producers have a narrow range of ingredients that can be used in formulating pig diets. Maize cobs, a by-product of a major cereal grown worldwide, have potential to be used as a pig feed ingredient. Maize cobs are either dumped or burnt for fuel. The major challenge in using maize cobs in pig diets is their lignocellulosic nature which is resistant to pigs' digestive enzymes. Pigs can however extract up to 25 % of energy maintenance requirements from fermentation products (Yen *et al*., 1991). In addition, dietary fibre improves pig health by promoting the growth of lactic acid bacteria (LAB), which suppress proliferation of pathogenic bacteria in the intestines; and reduces stress and behavioural problems (Montagne *et al*., 2003; de Leeuw & Ekkel, 2004; Kallabis & Kaufmann, 2012). All these benefits of fibre inclusion in growing pigs are not accounted for in diet formulations. Different approaches such as the use of exogenous enzymes and genetic selection have demonstrated the potential of improving utilisation of high fibre in pig diets.

The benefits of exogenous enzymes in improving nutrient digestion in pigs have been documented (Omogbenigun *et al*., 2004; Jones *et al*., 2010; Kerr & Shurson, 2013). Literature on the use of exogenous enzymes to reduce fibre levels in maize cobs is scarce, but there is potential to use them to augment ensiling in this regard. Although ensiling is the use of controlled fermentation to preserve a crop or material of high moisture content (McDonald *et al*., 1991), it reduced the levels of the fibre in maize cobs (Gatel *et al*., 1988; Millet *et al*., 2005; Khan *et al*., 2006). Addition of cell wall degrading enzymes to maize forage at ensiling improved the chemical characteristics of the resultant silages and reduced fibre content (Sheperd & Kung, 1996; Meeske *et al*., 1999; Colombatto *et al*., 2004). The mechanisms involved however are not clear and need to be explored.

Genetic selection can improve utilisation of high fibre diets in pigs but it involves complex traits such as feed intake, digestion, fermentation and absorption that are influenced by many loci. Indigenous pig breeds in Southern Africa and the Meishan from China digested fibrous diets better than Large White and Yorkshire breeds respectively (Ndindana *et al*., 2002; Kanengoni *et al*., 2004; Urriola & Stein, 2012). Frank *et al*. (1983) reported variability in pigs' abilities to utilize high level maize cob diets to an extent that the pigs could be separated into high, medium and low performance groups. In order to make genetic progress in traits linked to fibre utilisation it requires new selection criteria and strategies. The post genomic era with developments in proteomics and metagenomics has provided opportunities for using these techniques to solve pig production challenges. The 'omics' can be used to explain the mechanisms involved in the morphological and physiological responses in pigs fed on fibrous diets and help formulate in innovative ways more precise functional diets. Proteomics can evaluate thousands of proteins produced by tissues at a specific time and under any given conditions; such as pigs fed high fibre diets

(Bendixen *et al*., 2010; Picard *et al*., 2012; Tessitore *et al*., 2013). Unfortunately few published reports have applied proteomics in assessing the contribution of dietary fibre to pig growth and welfare.

Utilisation of high fibre diets in pigs can also be improved through improved fermentative processes in the gut. Kanengoni *et al*. (2002) hypothesized that the intestinal microbiome of indigenous pigs of Southern Africa is highly developed and complex, enabling them to effectively utilize fibrous feeds. However, only 1 % of gut microbial communities are culturable (Hugenholtz *et al*., 1998). Traditional methods that only study gut microbial diversity and ecology based on classical anaerobic culture techniques, would therefore not suffice in testing the above hypothesis. Identification of such microbes could provide an alternative to the use of exogenous enzymes to increase digestibility and energy supply to the pig from fibre-rich feeds. Metagenomics and sequencing technologies can identify and characterize microbes at the molecular level including those that cannot be cultured (Caporaso *et al*., 2011; Rastogi & Sani, 2011). This can lead to effective strategies in improving utilisation of high fibre diets. For example, Ziemer *et al*. (2012) demonstrated that using fibre-fermenting bacteria in a probiotic-like approach improved utilisation of high fibre diets.

Maize cobs are a valuable underutilized resource which if exploited as a pig feed ingredient could benefit pig producers and maize growers. There is need to develop effective technologies that can increase utilisation of fibrous ingredients such as maize cobs. Innovative maize cob processing methods and selection criteria for genetic improvement of pigs can be used in tandem to improve utilisation of the maize cobs. The presence of lactic acid bacteria (LAB) may inhibit exogenous enzymes' activities against structural carbohydrates, therefore ensiling of maize cobs with exogenous enzymes with a view of reducing fibre needs to be explored (Stokes, 1992; Xing *et al*., 2009). Proteomics can identify proteins whose expression or abundance is associated with enhanced fibre utilisation in pigs and consider them as biomarkers that could be used to predict the growth, intake or health status of the pigs (Picard *et al*., 2012). An understanding of the pig intestinal microbiome is needed to devise effective strategies of feeding fibrous diets. The South African Windsnyer-type Indigenous (SAWIP) and Large White x Landrace (LW x LR) pigs are two divergent breeds, kept commonly albeit by different sections of the South African pig industry. While the SAWIP is unimproved, grows slowly and lays down fat readily, it is hardy and survives on marginal levels of nutrition including fibrous diets. Many rural farmers keep this breed. The LW x LR is a prototype of the commercial breed, which is fast growing, lays down lean and requires high levels of nutrients for growth. The aim of this study was therefore to explore proteomics and microbiome profiling in the indigenous and commercial breeds of pigs fed high fibre diets and relate them to growth perfomance and nutrient digestibility parameters.

1.2 Objectives

The objective of the study was to explore the relationships between proteomic and microbiome profiles of South African pig genotypes and utilisation of high fibre diets

The specific objectives were to:

1. Determine the effect of ensiling maize cobs on nutrient quality, diet preferences and nutrient digestion by South African pig genotypes
2. Assess the growth performance, carcass traits and blood metabolite profiles of South African pig genotypes fed high fibre diets
3. Estimate relationships among proteomic profiles, nutrient digestibility and growth performance of South African pig genotypes fed high fibre diets; and
4. Profile the colon microbiome of South African pig genotypes fed high fibre diets

1.3 Hypotheses

The hypotheses tested were that:

1. Ensiling of maize cobs improves the intake and utilisation of diets
2. The proteomic profiles of indigenous pigs reflect an adaptation to high fibre diets
3. Specific proteins act as biomarkers that identify pigs with an enhanced ability to digest and utilise high fibre diets
4. Indigenous pig breeds have a myriad of structural carbohydrate digesting colon microbes which enhance fibre degradation

1.4 Layout of chapters

This dissertation consists of eight chapters; a general introduction and literature review followed by five research chapters and a general discussion and conclusion. Each chapter has its own abstract, introduction and references.

Chapter 1: General Introduction

This chapter provides a general introduction, motivation, objectives, and hypotheses and gives a description of the layout of the dissertation.

Chapter 2: Literature review

The literature review chapter gives an analysis of the use of maize cobs as a pig feed ingredient and their influence on the digestibility of nutrients, growth performance and intestinal microbial populations. It also sets the tone for the remainder of the dissertation by putting in perspective the value of maize cobs and

the potential use of proteomics and microbial profiling in evaluating the indigenous and commercial breeds of pigs.

Chapter 3: Effects of additives on the ensiling characteristics, nutrient composition and aerobic stability of maize cobs

Chapter 3 evaluates the effects of different silage additives on fermentation, aerobic stability and nutrient composition of ensiled maize cobs. The major findings were that ensiling maize cobs with molasses, whey and exogenous enzymes did not improve fermentation characteristics but exogenous enzymes reduced fibre fractions and energy content of maize cob silages. It provided guidance on the next chapter, which assessed the impact of including ensiled maize cobs in pig diets.

Chapter 4: Feed preference, nutrient digestibility and colon fermentation in growing South African Windsnyer-type Indigenous pigs and Large White x Landrace crosses fed diets containing ensiled maize cobs

This chapter analyses feed preference, nutrient digestibility and colon volatile fatty acid production in growing South African Windsnyer-type Indigenous pigs (SAWIP) and Large White x Landrace crosses (LW x LR) fed diets containing ensiled maize cobs. The SAWIP had higher preference for the CON diet compared to diets with maize cobs. There was no correlation between preference and diet digestibility in both breeds. The SAWIP digested nutrients better than the LW x LR in the high fibre diets. The need to understand further, how the SAWIP digested nutrients in high fibre diets better than the LW x LR led to investigation of the colon microbes in the two breeds in Chapter 5.

Chapter 5: Genomic analysis of intestinal microbial populations of South African Windsnyer-type indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) crosses fed diets containing ensiled maize cobs

Chapter 5 is a logical extension of Chapter 4 where metagenomic profiling explained the microbial response differentiation of the indigenous and commercial breeds of pigs fed ensiled maize cob based diets. There were no significant differences in the diversity of the core composition of gut bacterial communities between the breeds and diets. There were differences in the ratios of *Bacteroidia* to *Clostridia* between the SAWIP and LW x LR. Analysis of the microbiome revealed breed differences and not dietary differences. These findings together with those in Chapter 4 and other studies indicate differences in microbes in pig breeds in Southern Africa. This led to an investigation on the impact of these differences on metabolite profiles, liver histometry and in liver and serum proteomic changes in the pigs in Chapter 6.

Chapter 6: Serum metabolite profiles, liver histometry and proteomic analysis of South African Windsnyer-type indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) crosses fed diets containing ensiled maize cobs

In Chapter 6 serum metabolite levels and proteomics explained the metabolic response differentiation of the indigenous and commercial breeds of pigs fed maize cob based diets. Several proteins were upregulated in the LW x LR. There were differences in serum and liver proteins and in serum metabolite levels that were diet and breed related. Some of the upregulated proteins, *Guanidinoacetate N-methyltransferase*-like isoform 1 and *Catalase* explained the changes in metabolite levels. This suggests that proteomics can play a role in evaluating the performance of pigs under different feeding regimes. Further investigations on relationships of serum metabolites, histometry, and proteomic changes to the performance of the pigs led to Chapter 7.

Chapter 7: Growth performance and carcass characteristics of grower and finisher South African Windsnyer-type Indigenous and Large White x Landrace crossbred pigs fed diets containing ensiled maize cobs

This chapter analyses the growth performance and carcass characteristics when ensiled maize cobs were included in indigenous, and commercial breeds' pig diets. Although breed differences attributable to size existed with respect to the parameters evaluated, the SAWIP utilized the diets containing maize cobs better than the LW x LR. It demonstrated that ensiled maize cobs are a valuable feed resource to offset high feed costs given that their inclusion in pig diets did not affect negatively selected important commercial pork cuts.

Chapter 8: General discussion and conclusion

This chapter summarises the work done and develops the way forward based on the preceding chapters. In particular, it gives ideas on how best to use proteomics and microbiome profiling in evaluating breeds of pigs when feeding them high fibre diets. Selection of the better performing individuals based on biomarker identification is a possibility that should be pursued vigorously.

1.5 Research outputs and author contributions

The study generated the following peer review papers, manuscripts, conference outputs and conference posters:

1.5.1 Peer reviewed publications and manuscripts

1. Effects of silage additives on the ensiling characteristics, nutrient composition and aerobic stability of maize cobs. Manuscript under review at the South African Journal of Animal Science forms the basis of Chapter 3

2. Feed preference, nutrient digestibility and colon volatile fatty acid production in growing South African Windsnyer-type Indigenous pigs and Large White x Landrace crosses fed diets containing ensiled maize cobs. Manuscript published in Livestock Science Journal; Livestock Sci 171, 28–35, 2015 forms the basis of Chapter 4
3. Growth performance, blood metabolic responses and carcass characteristics of grower and finisher South African Windsnyer-type Indigenous and Large White x Landrace crossbred pigs fed diets containing ensiled maize cobs. Manuscript published in the Journal of Animal Science; J. Anim. Sci. 92:5739–5748, 2014, forms the basis of Chapter 7.

1.5.2 Conference outputs

1. Nutrient digestibility in growing South African Windsnyer-type Indigenous pigs and Large White x Landrace crosses fed diets containing ensiled maize cobs. **A.T. Kanengoni**, M. Chimonyo, B. Ndimba and K. Dzama. 47th Congress of the South African Society for Animal Science. 6th – 8th July 2014. University of Pretoria, Pretoria
2. The impact of enzyme inoculation on fermentation of ensiled maize cobs. **A.T. Kanengoni**, R.S. Thomas, B.D. Nkosi, B. Ndimba, M. Chimonyo, K. Dzama. Proceedings of the British Society of Animal Science and the Association of Veterinary Teaching and Research Work includes BSAS/EBLEX Workshop Improving Ewe Efficiency through Better Feeding. April 2014 Volume 5 Part 1
3. The impact of ensiled maize cob based diets on growth performance of South African pigs. **A.T. Kanengoni**, M. Chimonyo, B. Ndimba and K Dzama. 46th Congress of the South African Society for Animal Science. 23 – 26 June 2013. Odeion, University of the Free State, Bloemfontein
4. A comparison of preference patterns in South African local and commercial pigs fed ensiled maize cob based diets. **A.T. Kanengoni**, R. Thomas, M. Chimonyo, B. Ndimba and K. Dzama. 46th Congress of the South African Society for Animal Science. 23 – 26 June 2013. Odeion, University of the Free State, Bloemfontein
5. Effects of silage additives on the ensiling characteristics, nutritive value and aerobic stability of maize cobs. **A.T. Kanengoni**, R. Thomas, B.D. Nkosi , P. Ndou, M. Chimonyo, K. Dzama and Ndimba, B.at the 45th SASAS Congress in East London held from the 10th to the 13th July 2012

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Chapter 2

2 Literature Review

2.1 Introduction

Maize (*Zea mays L.*), a major cereal grown worldwide, generates cobs which can be used as pig feed ingredients (Frank *et al*., 1983; Kanengoni *et al*., 2004; Ndubuisi *et al.,* 2008). There have been efforts to use maize cobs as an animal feed because of the high competing demands with humans for the grain. Estimates of 180 to 200 kg of maize cobs produced per tonne of grains (Božović *et al*., 2004) translate to significant quantities of maize cobs being potentially available as feed resources. South Africa produced approximately 2.4 million tonnes of maize cobs during 2009/10 while the other major maize producers in sub-Saharan Africa; Nigeria, Tanzania and Malawi produced approximately 1.5, 0.9 and 0.7 million tonnes respectively (FAO, 2012).

Although maize cob usage is rarely accounted for, there have been efforts to evaluate possible applications in Kenya (Nangole *et al.,* 1983), Ghana (Tuah & Orskov, 1989), Tanzania (Urio & Katagile, 1987), Zimbabwe (Chimonyo *et al*., 2001; Mashatise *et al.,* 2005; Gadzirayi *et al*., 2006) and Nigeria (Omemu *et al.,* 2008; Akinfemi *et al.,* 2009; Opeolu *et al.,* 2009; Raheem & Adesanya, 2011). There are however currently no maize cob harvesting technologies and storage facilities at farm level. Farmers therefore tend to burn the maize cobs for heating and cooking, plough them back in the fields or throw them away, not only in sub-Saharan Africa (Urio & Katagile, 1987; Tuah & Orskov, 1989), but also in Asia and Eastern Europe (Latif & Rajoka, 2001; Božović *et al*., 2004; Zhang *et al*., 2010). This review will evaluate the physico-chemical composition and utilisation of maize cobs as ingredients in pig diets. Approaches to increase utilisation of maize cobs through ensiling using exogenous enzymes, and selection of pigs with enhanced ability to utilize high fibre diets using post-genomic technologies are then explored.

2.2 Physico-chemical properties of maize cobs

Maize cobs fall under the lignocellulose biomass classification; characterized by a close intertwining of cellulose (45 - 55 %), hemicellulose (25 - 35 %) and lignin (20 - 30 %) (Deutschmann & Dekker, 2012; Menon & Rao, 2012). A prerequisite to increasing maize cobs' incorporation into pig diets is a clear understanding of their physico-chemical properties because these have a direct bearing on gut fill, fermentation rate and overall digestion of diets. The physico-chemical properties of interest from a nutrition perspective are chemical composition, bulk density, viscosity, water solubility and water holding capacity (WHC).

2.2.1 Nutrient and chemical composition of maize cobs

Table 2.1 shows the dry matter, crude protein, ash, neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) of maize cobs. The crude protein, mineral composition and fat content of maize cobs are quite low and do not contribute much to the diets. The fibre components (NDF, ADF and ADL) are high and have been of much interest due to their effect on the digestibility and availability of other nutrients. A digestible energy value of 11 MJ/kg DM (Viljoen, 1993) and metabolizable energy value of 7 MJ/kg DM (Bredon *et al.,* 1987) have been reported.

Stage of maturity, cultivar, climate, soils and production methods affects composition of the maize cobs (Szyszkowska *et al*., 2007). Mature cobs tend to have higher NDF, ADF, dry matter (DM) and lower crude protein (CP) and starch than less mature cobs. Szyszkowska *et al*. (2007) reported that dry matter content in cobs was positively correlated with the content of starch, and negatively – with the content of NDF and ADF fractions. The cultivars tested did not differ in ADF, NDF and starch content in cobs; and the mineral composition depended on the cultivar, effective temperature sum and the farm type. Maize cobs separate into nutritionally distinct, different sized particles comprising of a hard or woody fraction and a soft fraction consisting of glumes, core, grain clippings and fine dust when ground. Božović *et al*. (2004) reported that 1 mm sized maize cobs had higher CP and ether extract and lower cellulose, hemicellulose, ADF and NDF than 3 and 2 mm particle sized maize cobs.

Table 2.1 Nutritional composition of maize cobs

Nutrient (g/kg DM)	**A**	**B**	**C**	**D**	**E**
Dry matter	908.3	885.2	900	-	-
Crude protein	38.9	32.6	25	17.2	37.5
Ether extract	5.68	-	6	12.3	6
Ash	76.7	72.6	26	13.3	38
Crude fibre	286.9	-	324	456.8	387
Nitrogen free extract	-	-	529	500.4	533
Neutral detergent fibre	706.3	929.8	-	-	813
Acid detergent lignin	168.8	-	-	116.7	44
Acid detergent fibre	515.8	573.2	-	570.1	421
Cellulose	347.0	-	-	453.4	358
Hemicellulose	190.5	179.6	-	135.4	392

A: (Akinfemi, 2010)

B: (Kanengoni *et al*., 2004; Chimonyo *et al*., 2001.)

C: (Bredon *et al.,* 1987)

D: (Nangole *et al*., 1983)

E: (Stanogias & Pearce, 1985a)

The maize cob holo-cellulose (the sum of cellulose and hemicellulose) proportion, ranges from 0.73 to 0.87 (Donnelly *et al*., 1973; Božović *et al*., 2004; Kumar *et al*., 2010a; 2010b). The holo-cellulose consists of α- and β-cellulose (composed of degraded cellulose) and γ-cellulose (consisting mainly of hemicelluloses) in proportions of 5.2:2.8:3.0 (Kumar *et al*., 2010a). Cellulose, a polysaccharide of alternating linear glucose units linked together by β - (1→4) - glycosidic bonds serves as the main structural component of the cob's cell walls. It is embedded in a matrix of lignin and hemicelluloses, which are branched hetero-polymers of pentoses (xylose, arabinose) and hexoses (glucose, mannose, galactose), sometimes substituted with uronic acids (or their O-methyluronic derivatives), acetyl groups and phenolic compounds in various proportions. In addition to providing structural support, hemicelluloses in certain situations act as seed storage carbohydrates (Scheller & Ulvskov, 2010). Half of maize cob hemicelluloses are xylans (Ebringerová & Heinze, 2000; Vázquez *et al*., 2006). Xylan is one of the hemicelluloses that has been studied extensively, owing to its diverse applications (Ebringerová & Heinze, 2000; Vázquez *et al*., 2006). Cellulose and xylans impute water-holding properties, which can result in considerable bulking of digesta (Ndou *et al.*, 2013).

2.2.2 Water solubility and bulk density

It is unclear how hemicelluloses crosslink with cellulose and lignin, and affect cellulose crystallinity and biomass degradation (Xu *et al*., 2012). These linkages however determine the water solubility and bulk density properties associated with maize cobs. Božović *et al*. (2004) reported that maize cobs' water solubility ranged from 4-11 % and water absorptive capacity ranged from 1.08 to 5.80 g/g depending on the size of particles. This compared well with Van Nevel *et al*. (2006) who reported water holding capacity of 4.6 g/g DM and a solubility of 4.5 %. In the study by Božović *et al*. (2004), the maize cobs were twice more soluble in sodium hydroxide than in water.

Pathak *et al*. (1986) compared the bulk density, equilibrium moisture content (EMC) at 80 % relative humidity and gross energy of maize cobs to other crop residues such as Arhar stalks, cotton sticks, maize stalks and rice straw. Maize cobs' bulk density (100 kg/m^3) was categorized as medium and had an EMC of 28 %. Residues having low bulk density present handling and storage problems, including poor flow properties. The EMC describes the moisture state of a hygroscopic material as it equilibrates on a moisture basis with its environment and has implications on shelf life (Igathinathane *et al*., 2005). Igathinathane *et al*. (2005) observed EMC ranges of 3.9 % to 56.4 %, 3.1 % to 41.1 %, and 2.7 % to 71.5 % for maize leaf, stalk skin, and stalk pith on a dry matter basis, respectively. They reported that the higher EMC values for maize stover leaf could result in a greater propensity for the onset of mould growth and therefore determined minimal storage requirements. Similar considerations should be applied when storing and using maize cobs since they have a relatively high EMC value and tend to get mouldy quickly increasing the risk of mycotoxicosis.

2.2.3 Viscosity

An increase in solubility of dietary fibre raises luminal viscosity and increases the water-binding capacity of digesta in the small intestine (Canibe & Bach Knudsen, 2002). Montagne *et al*. (2003) reported that the ability of a fibre substrate to increase viscosity of the intestinal digesta plays a very important role in its effect on the morphological characteristics of the epithelium in the intestinal tract. Van Nevel *et al*. (2006) reported a lower viscosity of maize cobs (1.01 mPa.s) compared to sugar beet pulp (1.67 mPa.s), wheat bran (1.13 mPa.s) and chicory roots (1.51 mPa.s). Feeding the different fibre substrates had no effect on viscosity of contents sampled in the jejunum, caecum and colon of weaner piglets.

2.3 Effects of maize cob supplementation on growth and digestive physiology

The value of the contribution of maize cobs when supplemented in pig diets is complex. It should encompass their effects on; feed intake, the digestibility of other nutrients, intestinal digestive physiology and on growth performance and not be measured only by the nutrients they contribute. A better understanding of these effects will assist in formulating more accurate and appropriate diets for pigs.

2.3.1 Effects of maize cob inclusion on feed intake and nutrient digestibility

It would be advantageous to be able to predict feed intake when feeding bulky feeds since they reduce nutrient density and digestibility. Ndou *et al*. (2013) reported that WHC, NDF and ADF contents of bulky feeds provide relationships with scaled feed intake (SFI) that can predict gut capacity in weaner pigs. In their study, feed intake in diets containing maize cobs at 80, 160, 240 g/kg levels was similar largely due to the low WHC of maize cobs.

Increases in maize cob level in the diet reduced the digestibility of organic matter (OM), DM, NDF, ADF, hemicellulose, nitrogen and energy (Table 2.2; Stanogias & Pearce 1985a; Kanengoni *et al*., 2002; Ndubuisi *et al*., 2008). An increased rate of passage and sequestration of nutrients in the fibre preventing their digestion explained the reduction in digestibility (Stanogias & Pearce, 1985a; Fevrier *et al*., 1992). This reduction was however, less in certain breeds like the Mukota, an indigenous pig from Southern Africa, and in older pigs (Table 2.2). In the Mukota pigs, digestibility of NDF decreased by 16 % while in the Large White x Landrace crosses (LW x LR) it decreased by 21 % when the inclusion level of maize cobs in the diet was increased from 100 to 300 g/kg (Kanengoni *et al*., 2002). Similarly, the digestibility of ADF reduced by 23 and 35 % for the Mukota and LW x LR crosses respectively as the maize cobs were increased from 100 to 300 g/kg in the diet. Explanations for the differences were that gut capacity and intestinal microbial populations differ among age groups (Varel & Pond, 1985; Kyriazakis & Emmans, 1995; Whittemore *et al.,* 2003; Thacker & Haq, 2009).

Table 2.2 Digestibility coefficients of dry matter (DM), neutral-detergent fibre (NDF), acid detergent fibre (ADF), hemicellulose (Hemi), and nitrogen (N) by pigs fed diets containing maize cobs (MC)

Breed and weight (kg)	**MC level**	**NDF level**	**DM**	**NDF**	**ADF**	**Hemi**	**N**	**Researchers**
LW		75	0.87	0.23	0.27	0.32	0.92	
(45)		150	0.81	0.10	0.18	0.02	0.89	Stanogias &
		225	0.76	0.21	0.24	0.20	0.88	Pearce,
		300	0.69	0.18	0.20	0.20	0.84	(1985a)
sem			0.008	0.031	0.036	0.032	0.007	
LW	0	80	0.88	0.62	0.69	0.58	0.86	
(70-87)	75	135	0.80	0.44	0.38	0.49	0.83	Frank *et al*.
	150	189	0.75	0.41	0.30	0.52	0.83	(1983)
sem			0.057	0.022	0.020	0.030	0.066	
LW x LR	0	276.4	0.87	0.87	0.68	0.91	0.87	
(75)	100	360.3	0.74	0.68	0.54	0.72	0.84	
	200	402.9	0.75	0.59	0.51	0.61	0.83	
	300	523.5	0.66	0.47	0.19	0.59	0.84	Kanengoni *et*
sem			0.014	0.027	0.034	0.034	0.012	*al*. (2002)
Mukota	0	276.4	0.86	0.86	0.69	0.90	0.86	
(33)	100	360.3	0.72	0.64	0.44	0.74	0.80	
	200	402.9	0.72	0.49	0.43	0.58	0.80	
	300	523.5	0.65	0.54	0.39	0.65	0.79	
sem			0.014	0.027	0.034	0.034	0.012	
LW x LR	0		85.8				86.4	
	50		82.2				84.2	Nduibisi *et al*.
	100		78.8				76.1	(2008)
	150		68.8				69.7	

MC, Maize cob; LW- Large White; LW x LR – Large White x Landrace

2.3.2 Effect of maize cob inclusion on growth performance

There is a general reduction in average daily gain and feed intake with increase in level of maize cob inclusion (Table 2.3; Frank *et al*., 1983; Ndindana *et al*., 2002; Kanengoni *et al*., 2004; Ndubuisi *et al*., 2008). Frank *et al*. (1983) reported individual variability in ability to utilize the higher level maize cob diets such that the pigs could be separated into distinct high, medium and low performance groups. The differences in responses to high fibre diets are due to genetic and physiological determinants of feed

intake (Frank *et al*., 1983). Similarly, the Mukota pigs demonstrated an ability to utilise maize cob diets better than commercial breeds (Ndindana *et al*., 2002; Kanengoni *et al*., 2004). In a growth performance study, the LW x LR crosses decreased growth rate by 26 % and the Mukota by 19 % as the maize cobs were increased from 0 to 300 g/kg in the diet (Kanengoni *et al*., 2004). There are no clear explanations for these differences.

Table 2.3 Growth performance of pigs fed diets containing different levels of maize cobs

Breed	**MC**	**NDF**	**Growth parameters**				**Researchers**
	level	**level**	**IW**	**ADG**	**ADFI**	**FCR**	
LW	0	80	30.2	0.84	2.09	0.401	Frank *et al*. (1983)
	75	135	30.4	0.81	2.21	0.370	
	150	189	30.5	0.79	2.33	0.341	
sem			0.4	0.01	0.03	0.005	
LW x LR	0	276.4	25	0.66	0.11	4.01	Kanengoni *et al*. (2004)
	100	360.3		0.60	0.11	3.76	
	200	402.9		0.55	0.11	3.84	
	300	523.5		0.49	0.11	4.34	
Mukota	0	276.4	14	0.36	0.12	5.59	
	100	360.3		0.32	0.10	4.56	
	200	402.9		0.25	0.11	5.41	
	300	523.5		0.29	0.11	6.25	
LW	0		19.4	0.56	1.54		Nduibisi *et al*. (2008)
	50		19.3	0.58	1.68		
	100		19.2	0.54	1.5		
	150		19.6	0.45	1.41		
sem			0.46	0.05	0.07		

MC, maize cobs; IW, Initial weight; ADG, average daily gain; ADFI, average daily food intake; FCR, food conversion ratio; LW, Large White; LR, Landrace; #ADFI was metabolic body weight ($kg/kg^{0.75}$)

An understanding of the adaptive mechanisms employed by the pigs which performed better could be used to better exploit fibrous agricultural by-products leading to either better diet formulation or more precise selection criteria or both. On the other hand, since Sub-Saharan Africa and the Asian sub-continent have significant populations of indigenous breeds raised by smallholder farmers, these results suggest that they can benefit from maize cob based diets (Chimonyo *et al*., 2005; Nidup & Moran, 2011). For commercial breeding stock, incorporating high fibre diets with maize cobs is a potentially economically viable proposition.

2.3.3 Effect of maize cob inclusion on intestinal volatile fatty acids and blood metabolites

The pigs' ability to extract up to 25 % of energy maintenance requirements from fermentation end products has been documented (Yen *et al.,* 1991). This derives from volatile fatty acids (VFA), which include mainly acetate, propionate and butyrate. The absorption of VFA's occurs in the colon and metabolism occurs in the liver and muscle. Systemically, VFA's influence changes in glycemia, lipidemia, uremia and nitrogen balance (Tungland & Meyer, 2002). Locally, VFA's have a trophic epithelial effect as butyrate is used primarily by the colonocytes, and provides a major source of energy for its metabolic activities (Guilloteau *et al*., 2010). Investigations on the fermentability of maize cobs produced variable results. Stanogias & Pearce (1985c) reported that diets supplemented with maize cobs resulted in similar VFA's concentrations to those having lupin hulls, wheat bran and lucerne stems in the proximal colon of pigs. In contrast, Van Nevel *et al*. (2006) reported that incubations with small intestinal contents did not degrade maize cobs in *in vitro* and only to a small extent in caecal incubations compared to sugar beet pulp, chicory roots and wheat bran. However, there was an increase in lactic acid concentration in the caecum and colon when the maize cobs were included in a diet fed *in vivo* (Van Nevel *et al*., 2006). They attributed this to high amounts of fibre from maize cobs reaching the hindgut and potentially being available for degradation and fermentation by the flora. The anomaly between *in vivo* and *in vitro* results points to other dynamics involved in fermentation in live animals that need investigation.

Frank *et al*. (1983) reported a linear decrease in glucose levels and an increase in urea levels in cross-bred pigs fed diets with incremental levels of 7.5 and 15 % maize cobs as fibre sources. The glucose decrease was attributed to less starch in the diet since maize cobs were incorporated in the diet at the expense of grain, while the high urea was because of increased ammonia production by intestinal microbes. In contrast, Mashatise *et al*. (2005) reported no differences in plasma glucose, urea and creatinine levels in Mukota and Mukota x Large White gilts fed a control and a high fibre diet with 20 % maize cobs. Breed of pig and level of maize cobs differentially affects serum energy metabolites similar to what type of fibre does as reported by Weber & Kerr, (2012). Measurements of serum concentrations of metabolites and hormones including cholesterol, non-esterified fatty acids, leptin and insulin need to be explored further.

2.3.4 Effect of maize cob inclusion on microbial flora

Feeding maize cob supplemented diets increased the number of total bacteria in the stomach and proximal jejunum marked by higher numbers of *Lactobacilli* and to a less extent an increase in *Bifidobacteria* (Van Nevel *et al*., 2006). There is need to investigate if there is a link to the fact that maize cob derived heteroxylans and xylo-oligosaccharides demonstrated immuno-stimulatory effects and prebiotic characteristics (Ebringerová *et al.,* 1995; Samanta *et al.,* 2012). Pig microflora contain highly active ruminal cellulolytic and hemicellulolytic bacterial species, which include *Fibrobacter succinogenes* (*intestinalis*), *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Butyrivibrio* spp., *Prevotella ruminicola*

and *Clostridium herbivorans* (Varel & Yen, 1997). The populations of these microorganisms increase in response to the ingestion of diets high in plant cell wall material. Urriola & Stein (2012) suggested that the superiority of the Meishan pig to digest insoluble fibre over the Yorkshire could be a result of differences in the microbial population or types. Further research is needed to determine the influence of breed on intestinal microbial populations.

2.3.5 Effect of maize cob inclusion on intestinal morphology

High fibre diets elicit hypertrophy of visceral organs, increase gastric and intestinal secretions and induce variable intestinal villous and cryptic changes in pigs depending on the fibre's physico-chemical characteristics, fibre level, feeding duration and breed or age of the pig (Montagne *et al*., 2003). Stanogias & Pearce (1985b) reported that prolonged intake of maize cobs supplemented-diets by growing pigs led to a hypertrophy and increased weight of segments of the gastrointestinal tract, similar to lupin hull, wheat bran, and lucerne stems supplemented diets. The responses were especially marked in the large intestine, and there were increases in mass or length, or both. However, level and type of protein, and particle size also similarly affected the morphology of the intestines (Brunsgaard, 1998). Anugwa *et al*. (1989) and Wenk (2001) pointed out that the hypertrophy of visceral organs in pigs fed high fibre diets increase the energy requirements of pigs due to the extra metabolic demand and nutrient needs for visceral organ development and maintenance.

Highly viscous diets increase proliferation of the villous and crypt cells by sloughing of the superficial layers translating to changes in the villous height/crypt depth ratio (Montagne *et al*., 2003). A low villous height/crypt depth ratio translated to low digestive capacity (Montagne *et al*., 2003). Van Nevel *et al*. (2006) reported an increase in the villous height/crypt depth ratio when feeding a maize cob supplemented-diet and suggested that it was due to an increased villus height. In addition, the numbers of intra-epithelial lymphocytes (IEL) were low in the maize cob supplemented diets indicating a lower renewal rate of the small intestinal mucosa (Van Nevel *et al*., 2006). Large numbers of the IEL in the villous epithelium indicate a faster renewal rate of the epithelium since they are responsible for clearing damaged or infected cells by apoptosis and cytolytic activity (Nishiyama *et al*., 2002). Understanding of molecular and cellular mechanisms regulating intestinal nutrient absorption allows the design of rational and innovative approaches to formulate feed and feed additives to ensure the health and well-being of the animal (Shirazi-Beechey *et al*., 2011).

2.4 Approaches to improving maize cob utilisation by pigs

To utilize maize cobs effectively as a feed resource, ways to improve their utilisation in pig diets should be devised. This can be done through improved processing, expanding the formulation objectives to

include functional properties like prebiotic properties and their potential health benefits and using pigs selected for better utilisation of high fibre diets.

2.4.1 Processing of maize cobs

One approach to improve utilisation of maize cobs by pigs is through the disruption of its cellulose, hemicellulose and lignin matrix using biological, mechanical and chemical means rendering them more digestible. Limited data are available on the effect of mechanical or chemical processing on changes in fibre utilization in pigs (Kerr & Shurson, 2013). Grinding, heating and fermentation of the feedstock in the ethanol production process modifies fibre in distilled dried grains with solubles (DDGS) making it more digestible than corn fibre (Le Gall *et al*., 2009; Urriola *et al*., 2010). Investigations on affordability and effectiveness of chemicals and exogenous enzymes, and ensiling to improve digestibility of maize cobs have been pursued.

2.4.1.1 Use of chemicals

Menon & Rao (2012) reviewed extensively the use of acid, alkali or organic acids to disrupt fibre matrices in lignocellulose biomasses. Concentrated and diluted acids work by breaking the rigid structure of the lignocellulosic material while alkali causes the degradation of ester and glycosidic side chains resulting in structural alterations of lignin, cellulose swelling and partial decrystallization of cellulose. Strong acids or bases disrupted lignocellulosic bonds in maize cobs and increased subsequent hydrolysis (Latif & Rajoka, 2001; Zhang *et al*., 2010). Božović *et al*. (2004) showed that maize cobs are highly soluble in sodium hydroxide. The treatment of maize cobs with chemicals such as ammonia and sodium hydroxide significantly reduced fibre levels (Tuah & Orskov, 1989). However, concerns on the use of corrosive substances and the dangers associated with handling chemicals limit their use. Instead, there have been growing trends to explore bio-fermentation processes and use of exogenous enzymes.

2.4.1.2 Use of exogenous enzymes

The addition of exogenous enzymes to animal feeds in efforts to improve nutrient digestion were reviewed in detail (Chesson, 1993; Bedford, 2000; Kerr & Shurson, 2013). Exogenous enzymes are increasingly being used in pig diets to offset anti-nutritional factors including fibre since pigs lack the appropriate digestive enzymes (Omogbenigun *et al*., 2004; Jones *et al*., 2010). The large diversity and concentration of chemical characteristics existing among plant-based feed ingredients, as well as interactions among constituents within feed ingredients and diets necessitate the use of exogenous enzymes to improve usage (Barletta, 2010; Kerr & Shurson, 2013). Improvements in nutrient digestibility and pig performance from adding exogenous enzymes to growing pig diets depends on understanding these characteristics in relation to enzyme activity. Studies on the use of exogenous enzymes with maize cobs are scarce, so there is need to identify or develop new enzymes that significantly reduce fibre levels in maize cobs. Since the main constituent of maize cobs are cellulose and xylan (Ebringerová & Heinze,

2000; Vázquez *et al*., 2006; Kumar *et al*. 2010a), different xylanases and cellulases should be investigated singly or as cocktails to investigate potential synergistic efficacy.

2.4.1.3 Ensiling

Although ensiling is essentially the use of controlled fermentation to preserve a crop or material of high moisture by creating anaerobic conditions (McDonald *et al*., 1991), it can also reduce the levels of the fibre in the maize cobs (Gatel *et al*., 1988; Millet *et al*., 2005; Khan *et al*., 2006; Rezaei *et al*., 2009). Silage fermentation is a dynamic process that requires good anaerobiosis and a low pH; parameters, which are unfortunately difficult to attain with maize cobs. The rapid metabolism of water-soluble carbohydrates (WSC) to lactic acid by lactic-acid-bacteria (LAB) normally results in a low pH (McDonald *et al.,* 1991). Rapid removal of air and a low pH prevents the growth of unwanted aerobic bacteria, yeasts and moulds that compete with beneficial bacteria for substrates (Bolsen *et al*., 1996; Kung *et al.,* 1998). Drier feedstocks such as maize cobs have poor compaction and retain air pockets. In addition, increase in the dry matter content of the feedstock curtails the growth of lactic acid bacteria and reduces the rate and extent of fermentation, since acidification occurs at a slower rate and the amount of total acid produced is less (Ashbell *et al.,* 1991). Failure to remove air quickly, commonly leads to high temperatures and prolonged heating.

Addition of cell wall degrading enzymes to maize forage at ensiling improved the chemical characteristics of the resultant silages and reduced fibre content (Sheperd & Kung, 1996; Meeske *et al*., 1999; Colombatto *et al.,* 2004). The use of ensiling with exogenous enzymes with a view of reducing fibre needs to be explored further. Identifying the correct enzymes effective under silage conditions and with a high specificity for maize cob associated fibre components and determining the optimum conditions for ensiling of maize cobs including possible additives and inoculants are critical areas that need addressing.

2.4.2 Evaluating pig responses at cellular level

Although the pigs' abilities to digest and utilise fibrous diets have been investigated using classical nutritional, biochemical and histological methods (Hedemann *et al*., 2006; Morel *et al*., 2006; Degen *et al*., 2007; Serena *et al*., 2008; von Heimendahl *et al*., 2010), it is still unclear how progress can be made for increased productivity. This is partly because the pig gut is a complex organ with an extensive neuro-endocrine system which secretes enzymes, immunological substrates, and houses numerous microbes, and responds to fibre and its fermentation products in diverse ways (Furness & Poole, 2012). The challenge has been in quantifying these responses and translating them to new recommendations of feeding fibrous diets.

2.4.2.1 Use of proteomics to predict pig responses

The use of proteomics to evaluate pig performance is strategic since proteins are central to almost every process occurring in the body (Bendixen *et al*., 2010; Picard *et al*., 2010; D´Alessandro *et al*., 2011; Zhang *et al*., 2013). Some metabolic substrates have the ability to regulate specific genes and proteins (Friedman & Halas, 1998; Shiraz-Beechey *et al*., 2011). For example, fatty acids act directly instead of through hormones or the nervous system (Pe´gorier *et al*., 2004) and dietary carbohydrates stimulate the release of gut peptides such as glucagon-like peptide (GLP-1 and 2), glucose dependent insulinotropic peptide (GIP) and serotonin (Shiraz-Beechey *et al*., 2011).

Twenty-two proteins related to energy metabolism; oxidative stress; and cell proliferation and apoptosis (cell death) were up-regulated whilst 19 other proteins were down-regulated in the jejunum of weanling piglets supplemented with ZnO (3,000 mg/kg Zn) compared with the control pigs (100 mg/kg Zn) (Wang *et al*., 2009). It was shown that ZnO supplementation improved the reduction-oxidation state and prevented apoptosis in the jejunum of weaning piglets, thereby alleviating weaning-associated intestinal dysfunction and malabsorption of nutrients. Zhong *et al.* (2011) showed that the conjugated linoleic acid (CLA) supplementation significantly influenced the abundance of proteins related to energy metabolism, fatty acid oxidation and synthesis, amino acid metabolism, defence, transport and other processes. They concluded that proteome changes observed in the *longissimus* muscle contributed to greater intramuscular lipid content in CLA-supplemented pigs. This finding could form the basis of recommending the inclusion of CLA in diet formulations in situations where intramusclar fat needs to be increased.

In another study, the pathological mechanisms of β-conglycinin, a soybean-derived allergen, in weanling piglets were evaluated (Chen *et al*., 2011). It was shown that β-conglycinin directly induced intestinal damage by depressing intestinal cell growth, damaging the cytoskeleton and causing apoptosis in the piglet intestine. This was reflected in the up-regulation of mitogen-activated protein kinase 8 and heat shock 60 kDa protein 1, which promotes pro-apoptosis, whilst chaperonin containing t-complex protein 1 subunits 5 (CCT5) and 8 (CCT8), valosin-containing protein (VCP) transitional endoplasmic reticulum AT-Pase, and heat shock 70 kDa protein 8, all of which are anti-apoptosis proteins, were down-regulated. These results provided new information on how β-conglycinin affects piglets, which was completely different from speculations that assumed β-conglycinin caused delayed hypersensitivity reactions in affected animals. Thus proteome analysis derived findings such as these provided insights into mechanistic aspects of how different nutrients affected specific tissues and growth in pigs, which in turn allow science based recommendations regarding their use in order to get beneficial outcomes.

2.4.2.2 Use of metagenomics to manipulate fibre fermenting microbes

The utilization of molecular techniques such as denaturant gradient gel electrophoresis (DGGE) and metagenomics has improved the analysis of complex intestinal microbial communities in humans and animals (Apajalahti *et al*., 2001; 2004; Van der Wielen *et al*., 2002; O'Flaherty & Klaenhammer, 2010; Kau *et al*., 2011; Gravitz, 2012). Identification and characterization of intestinal microbes can lead to an

analysis of their metabolic profiles and possible impact on the host. This could then be manipulated in such a way as to benefit the host. For example, Ziemer *et al* (2012) reported improved nutrient and fibre digestibility and decreased fecal output resulting from feeding a live bacterium to pigs. This can improve the producers' profitability and decrease the environmental footprint of pork production.

2.5 Conclusion

Maize cobs are underutilised as pig feed in sub-Saharan Africa, yet they are available to most farming households. They affect digestibility, digestive physiology and growth performance of pigs. Different approaches to improve utilisation of maize cobs include processing of the maize cobs using chemicals, exogenous enzymes and through ensiling, proteomics and metagenomics. This information can potentially be used to make practical feed formulating decisions. This can lead to timeous development of effective technologies and models that can be used to improve utilization of maize cobs. Information garnered can also be used as biomarkers to select animals with an innate ability to utilize fibre for breeding.

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Chapter 3

3 Effects of additives on the ensiling characteristics, nutrient composition and aerobic stability of maize cobs

Abstract

The study determined the effects of silage additives on fermentation, aerobic stability and nutrient composition of ensiled maize cobs. Five treatments: (i) control (maize cobs without additives - CON), (ii) maize cobs with sugarcane molasses only (MOL), (iii) maize cobs with a combination of sugarcane molasses and whey (MOW), (iv) maize cobs with a combination of sugarcane molasses, whey and exogenous enzyme at 0.5 g/kg maize cob mixture (ENZ1) and, (v) maize cobs with a combination of sugarcane molasses, whey and exogenous enzyme at 1 g/kg maize cob mixture (ENZ2) were ensiled in 1.5 L anaerobic glass jars over 32 days. On day 32, CON silage pH (4.2) was lower than the pH values of ENZ1 (4.5; $P < 0.05$) and ENZ2 (4.6; $P < 0.05$) silages. There were no differences in lactic acid levels between CON and MOL, MOL and MOW, MOW and ENZ1, ENZ1 and ENZ2 silages. Acetic acid levels were greater ($P < 0.05$) in ENZ1 than ENZ2 silages. Ammonia nitrogen (NH_3-N) levels averaged 25g NH_3-N/kg TN which is indicative of stable fermentation. Ammonia-N levels concentrations were greater ($P < 0.05$) in MOW (37.3 g/kg TN) than in MOL silages (18.4 g/kg TN). The MOL silages had greater dry matter (DM; 437 vs 413 g/kg; $P < 0.05$) and ash (28.2 vs 17.8 g/kg DM; $P < 0.05$) but lower gross energy (GE; 18.2 vs 18.5 MJ/kg DM; $P < 0.05$), digestible energy (DE; 10.5 vs 11.5 MJ/kg DM; $P < 0.05$) and acid detergent fibre (ADF; 440 vs 465 g/kg DM; $P < 0.05$) concentrations than the CON silages. Acid detergent fibre concentration was lower ($P < 0.05$) in MOW than ENZ1 silages. There was a linear increase in ash ($P < 0.05$) while GE, DE, α-amylase neutral detergent fibre (αNDF) and ADF decreased ($P < 0.05$), linearly as the enzyme level increased. After exposure to air, all the silages had similar pH values (CON = 8.2; MOL = MOW = ENZ1 = ENZ2 = 8.3). Molasses silage produced more carbon dioxide (CO_2) than CON and MOW ($P < 0.05$) silages. The ENZ1 silage produced more CO_2 ($P < 0.05$) than MOW silage. Ensiling maize cobs with molasses, whey and exogenous enzymes did not improve fermentation characteristics but exogenous enzymes reduced fibre fractions and energy concentration of maize cob silages.

Key words: silage additives, dietary fibre, volatile fatty acids, fermentation

3.1 Introduction

The use of maize cobs as a pig feed ingredient is deterred by high fibre (930 g neutral detergent fibre (NDF) /kg dry matter (DM); 573 g acid detergent fibre (ADF) /kg DM) and low protein levels (35.5 g crude protein (CP) /kg DM) (Viljoen, 1993; Ndindana *et al*., 2002; Kanengoni *et al*., 2004). High fibre increases rate of feed passage in the pig gut and sequestrates nutrients in the fibre matrix reducing their digestion (Stanogias & Pearce, 1985; Fevrier *et al*., 1992). Fermentation, exogenous enzymes, strong acids and bases disrupt the maize cob fibre matrix structure enabling further breakdown of fibre components to their monomeric constituents (Latif & Rajoka, 2001; Le Gall *et al*., 2009; Zhang *et al*., 2010a; Urriola *et al*., 2010). The use of strong acids and bases to improve maize cobs as pig feed is unattractive because of safety, environmental and economic concerns. Ensiling reduces fibre content in forages (Gatel *et al*., 1988; Khan *et al*., 2006; Rezaei *et al*., 2009) and could therefore improve maize cobs. Maize cobs are however difficult to ensile because they compact poorly and have low water soluble carbohydrate (WSC) concentrations. Lactic Acid bacteria (LAB) metabolize WSC to lactic acid, a critical acid in lowering silage pH (McDonald *et al.,* 1991). Sugarcane molasses and fresh cheese whey contain WSC and LAB and are effective silage additives (Khan *et al*., 2006; Bautista-Trijillo *et al*., 2009; Repetto *et al*., 2011). Khan *et al*. (2006) successfully ensiled urea treated maize cobs (913 g/kg DM) with acidified molasses and improved digestibility and nitrogen utilization in buffaloes. Fresh cheese whey and sugar cane molasses are readily and cheaply available to farmers making them attractive resources.

Although cell wall degrading enzymes improved chemical characteristics and reduced fibre content in maize and sorghum straw silages (Meeske *et al*., 1999; Colombatto *et al.,* 2004; Xing *et al*., 2009); how they did so is poorly understood. In principle, exogenous enzymes added to silages partially degrade fibre to fermentable WSC for use by LAB since these organisms cannot use fibre as an energy source (Eun & Beauchemin, 2007). However, the presence of LAB may inhibit exogenous enzymes' activities against structural carbohydrates in silage (Stokes, 1992; Xing *et al*., 2009). Xylans comprise up to 50 % of maize cobs' hemicelluloses (Ebringerová & Heinze, 2000; Vázquez *et al*., 2006). Commercial xylanases could degrade xylans under anaerobic conditions, leading to reduction in other fibre components thus improving the nutritive value of maize cobs. The objective of the study was to determine the effect of adding sugarcane molasses, fresh cheese whey and exogenous enzymes on the fermentability, nutrient composition and aerobic stability of maize cobs.

3.2 Materials and Methods

3.2.1 Treatments, experimental design and measurements

Maize cobs (920 g/kg DM) were collected from the Agricultural Research Council - Animal Production Institute fields (ARC-API, Irene, Gauteng, South Africa), and ground to pass through a 5 mm sieve. They were then treated with distilled water to achieve 60 g/kg moisture content. Sugarcane molasses syrup was obtained from Obaro®, a local dealer; and fresh whey from the cheese making factory at ARC-Irene; while

Porzyme 9302®, an enzyme containing endo-1,4-beta-xylanase activity of 8000 U/g was obtained from Danisco Ltd (Tsessebe Crescent, Midrand, South Africa). Sugarcane molasses syrup was diluted with warm water at a ratio of 1:2 and then sprayed evenly over the maize cobs at a rate of 100 ml/ kg. Fresh whey (4.7 x 10^5 total bacterial counts) was added at a rate of 50 ml/kg of maize cob. Two levels (0.5 and 1 g/kg maize cob) of the enzyme were included in two treatments. The treatments were: i) control (maize cobs without additive, CON), ii) maize cobs with sugarcane molasses only (MOL), iii) maize cobs with a combination of molasses and whey (100 ml molasses and 50 ml whey per kg maize cobs, MOW), iv) maize cobs with a combination of molasses and whey (as in MOW) with exogenous enzyme at 0.5 g/kg maize cob mixture (ENZ1) and, v) maize cobs with a combination of molasses and whey (as in MOW) with exogenous enzyme at 1 g/kg maize cob mixture (ENZ2). These mixtures were ensiled in 1.5 L anaerobic glass jars (J. Weck, GmBHu. Co., Wehr-Oflingen, Germany) equipped with lids to enable gas release and kept at between 24 and 28 °C. Triplicate jars per treatment were opened on days 0, 1, 4, 15 and 32 of ensiling and sampled for the determination of pH and DM. Further, analyses for gross energy (GE), crude protein (CP), ether extract (EE), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were done on samples collected on days 0, 15 and 32. Analyses for water-soluble carbohydrates (WSC), volatile fatty acid (VFA) concentrations, lactic acid (LA) and ammonia-N (NH_3-N) were done on samples collected on days 0 and 32. After 32 days ensiling, silage samples were subjected to an aerobic stability test, whereby samples were exposed to air for five days and carbon dioxide (CO_2) production and pH were determined following the procedure of Ashbell *et al*. (1991).

3.2.2 Chemical analyses

To follow the fermentation dynamics during ensiling a 40 g sample of ensiled sample was collected from each jar and mixed with 360 ml of distilled water in a stomacher bag, homogenized and left for 24 h at 10 °C (Suzuki & Lund, 1980). It was then homogenized for 4 min and filtered through a Whatman No. 4 filter paper (G.I.C. Scientific, Midrand, South Africa) and the extract was used for determination of pH, WSC (Dubois *et al*. 1956), LA (Pryce 1969), VFAs (Suzuki & Lund, 1980) and NH_3-N (AOAC, 1990; ID 941.04). The DM of silage was determined by drying the samples at 60 °C until a constant mass was achieved and corrected for loss of volatiles using the equation of Porter & Murray (2001). Dry samples were ground through a 1 mm screen (Wiley mill, Standard Model 3, Arthur H. Thomas Co., Philadelphia, PA, USA) for chemical analyses. The αNDF was determined following the procedures of Van Soest *et al*. (1991) using heat stable α-amylase, and the ADF and ADLsa(sulphuric acid) concentrations were determined using the Fibretec System equipment (Tecator LTD., Thornbury, Bristol, UK). Acid detergent lignin (sa) was determined by solubilisation of cellulose with sulphuric acid. Separate samples were used for ADF and aNDF analyses and both included residual ash. Crude protein (ID 968.06), ash (ID 942.05) and EE (ID 963.15) were determined according to the procedures of AOAC (1990). The GE was determined with bomb calorimetry (MS-1000 modular calorimeter, Energy Instrumentation, Centurion, South Africa). Digestible energy (DE) of the silage was calculated from the formula;

DE (kcal/kg DM) = 4,168 - (91 x Ash %) + (19 x CP %) + (39 x EE %) - (36 x NDF %);

NRC (2012) adapted from Noblet & Perez (1993); and converted to MJ/kg DM by multiplying by a constant (0.0041868).

3.2.3 Statistical analyses

Data for fermentation, aerobic stability and nutrient composition of the silage were analysed for effects of treatment using General Linear Model (GLM) procedures of SAS (SAS Inst. Inc., Cary, NC). The effect of increasing the level of exogenous enzyme on DM, GE, DE, ADF, NDF and ADL levels was evaluated using PROC REG of SAS (SAS Inst. Inc.,Cary, NC). All data were tested for normality and homogeneity and comparisons were made to the 95 % significance level. The NH_3-N and lactic acid data were inverse transformed to achieve normality. The model used was;

$$Y_{ijk} = \mu + T_i + D_j + (Tx\ D)_{ij} + \varepsilon_{ijk}$$

where Y_{ij} is the dependant variable, μ is the overall mean, T is the treatment effect (i = CON, MOL, MOW, ENZ1, ENZ2), D is the day of ensiling (0, 15, 32), T x D is the interaction of treatment and day and ε_{ij} is the experimental error. The PDIFF statistic of SAS (SAS Inst. Inc., Cary, NC) was used to separate the treatment means. The effect of adding molasses was studied by comparing CON with MOL. The effect of adding whey was analysed by comparing MOL and MOW and the effect of adding the exogenous enzyme was studied by comparing MOW and ENZ1.

3.3 Results

Nutrient concentrations, water soluble carbohydrate level and pH in maize cobs with or without additives before ensiling are shown in Table 3.1. Changes in pH values of the CON, MOL, MOW, ENZ1 and ENZ2 silages for days 1, 4, 15 and 32 are shown in Figure 3.1. Control silage pH decreased rapidly from 6.3 on day 0 to 4.1 on day 15 at a greater rate than in MOL (6.0 - 4.9), MOW (6.1 - 4.7), ENZ1 (6.1 - 5.1) and ENZ2 (6.2 – 5.2) silages. At day 32, CON silage pH (4.2) was lower than the pH values of ENZ1 (4.5; $P < 0.05$) and ENZ2 (4.6; $P < 0.05$) silages. Volatile fatty acid, lactic and acetic acid and NH_3-N concentrations in the silages after 32 days are shown in Table 3.2. Butyric acid concentrations were not detectable at 32 days. There were no differences in LA concentrations ($P > 0.05$) between CON and MOL, MOL and MOW, MOW and ENZ1, ENZ1 and ENZ2 silages. Acetic acid concentrations were greater ($P < 0.05$) in ENZ1 than ENZ2 silages. Ammonia nitrogen (NH_3-N) concentrations averaged 25±6.6 g NH_3-N/kg Total Nitrogen (TN). Ammonia-N levels concentrations were greater ($P < 0.05$) in MOW (37.3 g/kg TN) than in MOL silages (18.4 g/kg TN).

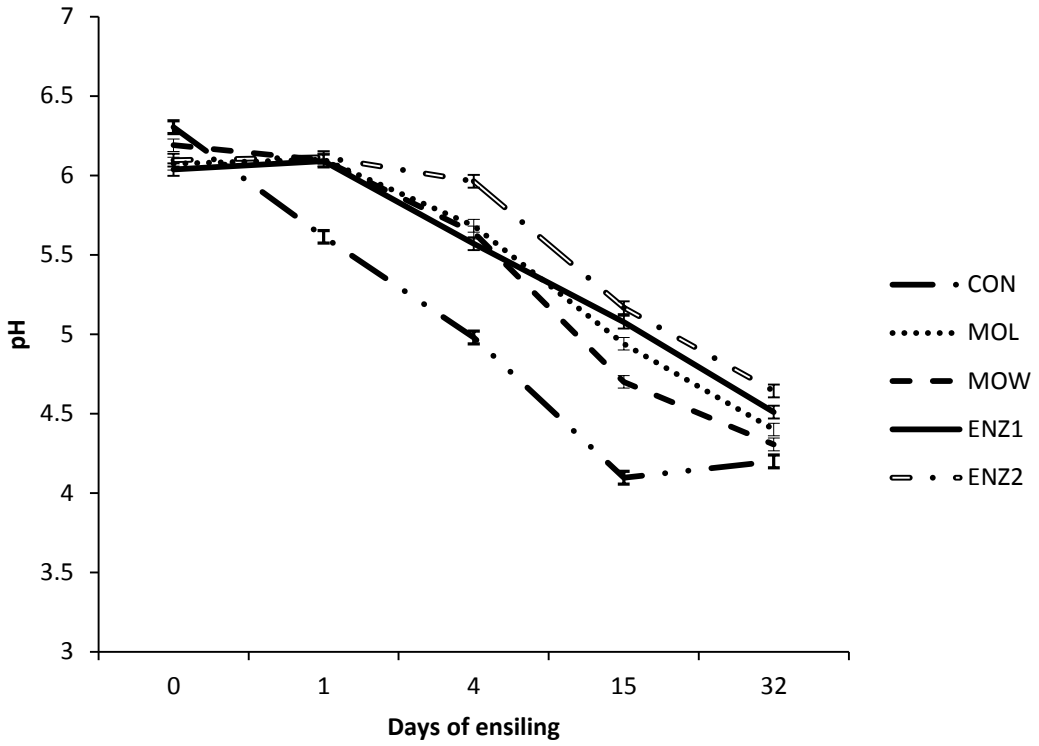

Figure 3.1 Changes in pH of CON, MOL, MOW, ENZ1 and ENZ2 silages at days 0, 1, 4, 15 and 32 days

Table 3.1 Nutrient and fibre, water soluble carbohydrate concentrations and pH in maize cobs with or without additives before ensiling (n=3)

Treatment	DM	GE	DE	CP	EE	Ash	αNDF	ADF	ADLsa	WSC	pH
	g/kg	MJ/kg DM	MJ/kg DM	g/kg DM							
CON	583	18.2	11.5	31	5.1	17.1	825	455	7.5	8.2	6.3
MOL	537	18.0	9.8	34	5.6	28.3	774	431	8.5	30.0	6.0
MOW	557	18.0	10.3	40	5.8	30.4	753	409	10.1	43.9	6.1
ENZ1	581	18.0	10.2	33	5.1	28.6	764	420	8.6	42.9	6.1
ENZ2	578	17.6	9.1	42	6.0	33.4	717	385	8.9	44.2	6.2

DM - dry matter; GE - gross energy; DE - digestible energy; CP - crude protein; EE - ether extract; αNDF- α-amylase treated neutral detergent fibre; ADF - acid detergent fibre; ADLsa - acid detergent lignin sulphuric acid determination

DE (kcal/kg) = 4,168 - (91 x % Ash) + (19 x % CP) + (39 x % EE) - (36 x % NDF); NRC (2012) adapted from Noblet & Perez (1993) and converted to MJ/kg DM by multiplying by a constant (0.0041868).

Table 3.2 Lactic acid, acetic acid and ammonia nitrogen (NH_3N) in maize cobs ensiled with or without additives after 32 days

Parameter	Treatment						[b]Probability			
	CON	MOL	MOW	ENZ1	ENZ2	RSD	CON Vs MOL	MOL vs MOW	MOW vs ENZ1	ENZ1 vs ENZ2
LA g/kg DM	1.1	0.9	1.0	1.0	0.77	0.14	0.185	0.378	0.590	0.265
AA g/kg DM	15.1	17.7	30.2	17.7	3.4	9.38	0.705	0.103	0.102	0.049
[a]NH_3-N g/kg TN	28.1	18.4	37.3	26.7	16.5	6.60	0.203	0.036	0.170	0.182

n=3
[a]NH_3-N g/kg TN - ammonia nitrogen g per kg total nitrogen; LA - lactic acid; AA - acetic acid; CP-crude protein; CON (control, maize cobs with no additives), MOL (maize cobs with molasses only), MOW (maize cobs with molasses and whey), ENZ1 (maize cobs with molasses, whey and enzyme at 0.05 g/kg); ENZ2 (maize cobs with molasses, whey and enzyme at 1 g/kg); RSD - residual standard deviation
[b]Probability of the contrasts CON vs MOL, MOL vs MOW, MOW vs ENZ1, ENZ1 vs ENZ2

Nutrient concentrations of the silages are shown in Table 3.3. There were greater ($P < 0.05$) DM and ash ($P < 0.05$) and lower ($P < 0.05$) GE, DE and ADF concentrations in the MOL silages than in the CON silage. Dry matter of MOW silage was greater than that of MOL and ENZ1 silages ($P < 0.05$).

Table 3.3 Nutrient and fibre concentrations in maize cobs with or without additives ensiled for 32 days (n=3)

Treatment	DM	GE	[a]DE	CP	EE	Ash	αNDF	ADF	ADLsa
	g/kg	MJ/kg DM		g/kg DM					
CON	413	18.5	11.5	30.2	4.7	17.8	828	465	79.1
MOL	437	18.2	10.5	31.7	5.5	28.2	790	440	86.7
MOW	445	18.1	10.1	35.7	6.1	30.2	768	425	73.1
ENZ1	435	18.2	10.6	32.0	5.2	30.0	798	455	77.3
ENZ2	436	18.1	10.3	34.1	5.7	33.1	788	436	99.4
RSD	2.18	0.10	0.46	4.80	0.58	3.28	20.3	13.2	9.10
[b]Probability									
CON vs MOL	<.0001	0.004	0.018	0.66	0.063	0.0004	0.032	0.031	0.443
MOL vs MOW	0.0002	0.158	0.254	0.251	0.182	0.414	0.192	0.160	0.196
MOW vs ENZ1	<.0001	0.433	0.204	0.286	0.056	0.933	0.101	0.019	0.663
ENZ1 vs ENZ2	0.812	0.191	0.478	0.540	0.318	0.195	0.602	0.147	0.060

DM - dry matter; GE - gross energy; DE - digestible energy; CP - crude protein; EE - ether extract; αNDF - α-amylase treated neutral detergent fibre; ADF - acid detergent fibre, ADLsa - acid detergent lignin, sulphuric acid determination
[a]DE (kcal/kg) = 4,168 - (91 x % Ash) + (19 x % CP) + (39 x % EE) - (36 x % NDF); NRC (2012) adapted from Noblet & Perez (1993) and converted to MJ/kg DM by multiplying by a constant (0.0041868).
CON (control, maize cobs with no additives), MOL (maize cobs with molasses only), MOW (maize cobs with molasses and whey), ENZ1 (maize cobs with molasses, whey and 0.05 g/kg enzyme); ENZ2 (maize cobs with molasses, whey and 1g/kg enzyme)
[b]Probability of the contrasts CON vs MOL, MOL vs MOW, MOW vs ENZ1, ENZ1 vs ENZ2

Linear regression coefficients of enzyme level and energy, ash and fibre concentrations in maize cobs ensiled for 32 days are shown in Table 3.4. There was a linear increase in levels of Ash ($P < 0.05$). There

were linear decreases (P < 0.05) in GE, DE αNDF and ADF as the enzyme level increased. Aerobic stability of the maize cob silages are shown in Table 3.4. There were no differences in pH among the silages (P > 0.05) on day 36. Molasses silage produced more (P < 0.05) carbon dioxide (CO_2) than CON (and MOW silages. The ENZ1 silage produced more CO_2 (P< 0.05) than MOW silage.

Table 3.4 Linear regression coefficients (± se) of enzyme level and energy, ash and fibre concentrations in maize cobs ensiled for 32 days

Parameters	Intercept	Reg Coeff	RSD	Linear ProbabilityP value	R^2
GE MJ/kg DM	18.1	-0.23	0.13	0.005	0.26
DE MJ/kg DM	10.3	-0.85	0.69	0.014	0.19
Ash g/kg DM	29.7	5.39	4.31	0.028	0.15
αNDF g/kg DM	779.2	-39.00	31.6	0.022	0.17
ADF g/kg DM	432.4	-32.67	24.4	0.016	0.19
ADL (sa) g/kg DM	70.2	26.24	9.9	0.056	0.55

n =3
DM - dry matter; CP- crude protein; GE - gross energy; DE-digestible energy; EE - ether extract; αNDF- α-amylase treated neutral detergent fibre; ADF - acid detergent fibre; ADL- acid detergent lignin
Reg Coeff – Regression Coefficient

Table 3.5 Carbon dioxide (CO_2) produced g/kgDM and pH changes of maize cob silages exposed to air for 5 days

	CON	MOL	MOW	ENZ1	ENZ2	SD	CON vs MOL	MOL vs MOW	MOW vs ENZ1	ENZ1 vs ENZ2
CO_2 g/kg DM	0.7	1.5	0.9	1.6	1.4	0.21	0.001	0.008	0.004	0.370
pH	8.2	8.3	8.3	8.3	8.3	0.07	0.143	0.858	0.721	0.766

CON (control, maize cobs with no additives), MOL (maize cobs with molasses only), MOW (maize cobs with molasses and whey), ENZ1 (maize cobs with molasses, whey and enzyme at 0.05 g/kg); ENZ2 (maize cobs with molasses, whey and enzyme at 1 g/kg)

3.4 Discussion

Maize cobs were mixed with water, molasses, whey and exogenous enzymes to reduce dry matter content, improve the water-soluble carbohydrate content and break down the fibre matrix. Dry matter for all treatments on day 0 (567 g/kg DM) was high compared to 500 g/kg DM reported by Khan *et al*. (2006) for urea treated maize cobs ensiled with enzose, acidified and non-acidified molasses and water and 400 g/kg DM for freshly chopped maize reported by Nkosi *et al*. (2009). Such a high DM impairs effective ensiling by curtailing growth of lactic acid bacteria and reducing the rate and extent of fermentation (Ashbell *et al.,* 1991). The low WSC content (8.2 g/kg DM) of the CON silage that was obtained pre-ensiling justified the addition of molasses and whey given that the other silages (MOL, MOW and ENZ 1 and 2) had WSC content of 30 g/kg DM and above. The WSC are fermented to lactic acid by epiphytic LAB, and a minimal

concentration of 30 g WSC/kg DM is critical for successful fermentation (Haigh & Parker, 1985; Weinberg *et al*., 1988; Bautista-Trijillo *et al*., 2009).

The results indicate that molasses, whey and enzymes are not necessary for quick reduction of pH when ensiling maize cobs. McDonald *et al*. (2002) reported that well-preserved silages had a pH range of 3.8 to 4.2 and only the CON silage attained that. There is no clear explanation why there were no differences in lactate levels between the CON and the other silages, which had been supplemented with molasses and whey. Zobell *et al*. (2004) stated that good silage has lactic acid levels ranging from 30 to 140 g/kg DM. Kung and Shaver, (2001) described a high quality silage as having a lactic acid concentration of at least 65 to 70 % of the total silage acids. Therefore, fermentation of silages in the current study was poor because of low lactic acid concentrations (0.77 to 1.0 g/kg DM) which were also less than 65 % of total acids. This concurs with Van Nevel *et al*. (2006) who reported poor *in vitro* fermentability of maize cobs. Zhang *et al*. (2010b) stated that a 2:1 ratio of lactic acid to acetic acid is an indicator of strong homolactic fermentation. Since lactate: acetate ratio (LA:AA) was less than 2:1 it suggests that the maize silages underwent hetero-fermentation. A likely explanation is the finding that addition of molasses to the wilted lucerne increased the acetate concentration and decreased the lactate to acetate ratio as reported by Hashemzadeh-Cigari *et al*. (2011). High concentrations of acetic acid could have been a result of poor compaction and retention of air pockets, a characteristic commonly associated with drier feedstocks like maize cobs (Kung & Shaver, 2001). Addition of homolactic inoculants can decrease the LA:AA ratio and may benefit the ensiling of maize cobs. The low ammonia concentrations observed in this study, average 25±6.6 g NH_3-N/kg TN, are indicative of minimal protein breakdown during the ensiling process. High concentrations of ammonia (>12 to 15 % of CP) are a result of excessive protein breakdown caused by a slow drop in pH or clostridial action (Kung & Shaver, 2001).

Addition of molasses to maize cobs was expected to increase the energy content but instead it resulted in lower gross and digestible energy content in the MOL silages. This can be explained by the fact that molasses have a high ash content which diluted the energy levels. Increases in ash, EE and ADL concentrations as level of enzyme increased corresponded to decreases in GE, DE, NDF and ADF concentrations and are suggestive of fermentative processes. The findings agree with reports that cell wall degrading enzymes reduced fibre content in maize forage at ensiling (Sheperd & Kung, 1996; Meeske *et al*., 1999; Meeske *et al*. 2002; Colombatto *et al.,* 2004).

The aerobic stability study was inconclusive. The CO_2 production (0.7 – 1.6 g/kg DM) obtained in the current study is an indication of DM losses and the extent of aerobic stability (McDonald *et al*., 1991). The aerobic stability of the maize cobs deteriorated with the addition of molasses and enzymes and stabilized with addition of whey. Nkosi *et al*. (2009) obtained higher CO_2 values for whole maize crop. There was however a similar increase in pH in all silages after exposure to air which is indicative of poor aerobic stability. Ensiling using homolactic fermentative inoculants could help improve aerobic stability.

3.5 Conclusions

The addition of molasses, whey and exogenous enzymes when ensiling maize cobs did not improve the fermentation quality. The use of exogenous enzyme at higher inclusion levels during the ensiling of maize cobs reduced the fibre fractions of the silage even though it was at the expense of energy content of the silages. It was concluded that ensiling maize cobs with molasses, whey and xylanase, as additives do not lead to a satisfactory balance of lower pH and fibre levels and higher digestible energy. Further work is needed to investigate the impact of diets containing ensiled maize cobs on feed preference, digestibility of nutrients and fermentability.

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Chapter 4

4 Feed preference, nutrient digestibility and colon fermentation in growing South African Windsnyer-type Indigenous pigs and Large White x Landrace crosses fed diets containing ensiled maize cobs

Abstract

A study was conducted to determine feed preference, nutrient digestibility and colon fermentation in growing South African Windsnyer-type Indigenous pigs (SAWIP) and Large White x Landrace crosses (LW x LR) fed diets containing ensiled maize cobs. Three treatments; CON (control diet without maize cobs), LMC (low maize cob level diet) and HMC (high maize cob level diet) were evaluated. Preference was assessed in 64 LW x LR (45 ± 6.7 kg) and 30 SAWIP (21 ± 3.6 kg) by offering the pigs choices between a common reference diet and a test diet. Apparent total tract digestibility (ATTD) and colon fermentation were determined using 15 LW x LR and 15 SAWIP pigs weighing 70 ± 6.9 kg and 49 ± 8.1 kg respectively. There were breed by diet interactions ($P < 0.05$) in period 1 (P1; 0-3 days) for preference of diets but none in period 2 (P2; 3-6 days). The two breeds' preferences for the LMC and HMC diets were lower ($P < 0.05$) than the 50 % preference index in P1. In period 2 (P2), the LW x LR preference values for LMC and HMC diets were lower ($P < 0.05$) than the 50 % preference index while the SAWIP's were not. The ATTD coefficients of crude protein (CP), acid detergent fibre (ADF), hemicellulose (Hemi) and neutral detergent fibre (NDF) in the HMC were greater ($P < 0.05$) than in the CON. The SAWIP had greater ($P < 0.05$) NDF digestibility coefficients than the LW x LR. There were breed x diet interactions ($P < 0.05$) for proportions of isobutyric acid (IBA) and butyric acid (BA), IBA concentration and acetic to butyric acid (AA:BA) and BA:IBA ratios. The LW x LR had greater concentrations of total volatile fatty acids (VFA) ($P < 0.05$) than the SAWIP. Ensiled maize cobs added to pig diets at 200 g/kg improved digestibility of nutrients. The differences in breed responses to the high fibre diets need further investigations to understand the mechanisms involved.

Keywords: fermentation, fibre, genotypes, palatability

4.1 Introduction

Ensiling reduces non-starch polysaccharide (NSP) levels in ingredients (Meeske *et al*., 1999; Khan *et al*., 2006; Rezaei *et al*., 2009). Farmers can find it easy to adopt and use this technology. In Chapter 3, ensiling reduced NDF and ADF levels in maize cobs thus overcoming one of the main deterrents to using maize cobs in pig diets. Ensiling is also expected to improve palatability and hence preference in feedstuffs from the volatile fatty acids (VFA) and organic acids produced. Preference is the intake of the test diet as a percentage of the total intake and is not generally considered when formulating diets for pigs yet it is a critical component of any feed and directly affects performance (Sola-Oriol *et al*., 2009a, b). Pigs demonstrated preferences for feeds based on texture, particle size, nature of feedstuffs, inclusion rate and freshness (Sola-Oriol *et al*., 2009a; 2011; Seabolt *et al*., 2010).

South African resource poor farmers keep indigenous breeds, the South African Windsnyer-type Indigenous pigs (SAWIP) under marginal conditions and they are often fed fibrous diets and nothing has been documented of their feeding behaviour except for anecdotal evidence. It would be important to determine if there are breed differences on the preferences for fibrous diets. The objective of the current study was therefore to assess feed preference, nutrient digestibility and colonic fermentability when ensiled maize cobs were included in indigenous and commercial pig diets.

4.2 Materials and methods

4.2.1 Ensiling process and diets

Maize cobs (920 g/kg DM) were collected from the Agricultural Research Council - Animal Production Institute fields (ARC-API, Irene, Gauteng, South Africa), and ground to pass through a 10 mm sieve. They were mixed with distilled water to lower the dry matter (DM) to 350 g/kg, and ensiled by compacting in 210 L drums lined with a plastic bag, and closed with a rubber lid to prevent damages to the bags by rodents. A packing density of 822 ± 33.5 kg/ m^3 was obtained and the drums were stored at between 22 and 29 ^{0}C. After 3 months of ensiling, drums were opened and sampled for the determination of fermentation characteristics and chemical compositions as described in Chapter 3, Section 3.2.2 (Table 4.1). The silage was then used to formulate diets with inclusion levels of 100 g and 200 g maize cob/kg of diet (as fed) as shown in Table 4.2. The diets were formulated to provide 14 MJ/kg digestible energy (DE), 180 g crude protein (CP)/kg DM and 11.6 g lysine /kg which meet and exceed the requirements of growing pigs (NRC, 1998). This resulted in three treatments namely; control diet without maize cobs (CON), diet containing 100 g maize cobs/kg diet (LMC), and diet containing 200 g maize cobs/kg diet (HMC). The silage drums were opened weekly as the diets were mixed to prevent spoilage. The effect of these diets on feed preference, nutrient digestibility and colonic fermentation in growing pigs were evaluated in two experiments. The experimental procedures described in the study were approved by the Animal Ethics Committee of the Agricultural Research Council, Animal Production Institute (ARC-API).

Table 4.1 The fermentation characteristics and chemical composition of ensiled maize cobs at 90 days

Fermentation characteristics	Quantity
Dry matter, g/kg	395.0
pH	4.0
Water soluble carbohydrate, g/kg DM	5.1
Lactic acid g/kg DM	19.3
Acetic acid, g/kg DM	49.5
Propionic acid, g/kg DM	ND
Butyric acid, g/kg DM	ND
Composition	
Organic matter, g/kg DM	923.4
Gross energy, MJ/kg DM	18.1
Crude protein, g/kg DM	41.2
Ether extract, g/kg DM	8.7
Acid detergent fibre, g/kg DM	459.5
αNeutral detergent fibre, g/kg DM	773.5

ND – Not Detected

4.2.2 Experiment 1 Effect of diets containing ensiled maize cobs on feed preference

4.2.2.1 Animals, housing and experimental design

Sixty four Large White × Landrace crossbred pigs (LW x LR) weighing 45 ± 6.7 kg live body weight and 30 South African Windsnyer-type Indigenous pigs (SAWIP) weighing 21 ± 3.6 kg, balanced on sex were randomly selected from the ARC-Irene pig breeding units and used in double choice experiments as described by Sola-Oriol *et al*. (2009a). As the SAWIP and LW x LR differ in their mature body size (300 – 350 kg vs 140 - 180 kg), growing pigs of a similar degree of maturity (0.15 of adult body weight) were chosen from each breed. The pens for the LW x LR measured 2 x 1.5 m and the SAWIP's were 1.5 × 0.9 m in environmentally controlled houses with the temperature ranging from 22 to 25 °C. The pigs stayed individually in pens containing two identical feeders (placed side by side). The feeders were checked and adjusted twice each day to ensure constant access to fresh feed and minimize any possible wastage. The pigs were blocked by weight and breed when assigned to the preference treatments. One feeder in each pen contained a diet without maize cobs (CON) and acted as the reference diet and the other feeder contained a test diet with either 0 (CON), 100 (LMC) or 200 (HMC) g/kg ensiled maize cobs resulting in three preference comparisons. In each group and experimental period, the two-way comparisons consisting of a CON diet vs CON diet comparison which was used as control were included to validate proper control and ensure that factors such as feeder placement, temperature and ventilation differences were not confounding (Seabolt *et al*., 2010). The remaining two two-way comparisons tested the level of fibre on preference. There were at least 21 replications for the LW x LR for each comparison and 10 for the SAWIP. The study was carried out in six days in two periods of 0-3 and 4-6 days. One-half of the feeders containing the reference diet were positioned on the left side of the pen and one half on the right side of the pens. Water was freely available through low- pressure nipple drinkers.

Table 4.2 Composition of experimental diets; control (CON), low maize cob inclusion (LMC) and high maize cob inclusion (HMC) fed to growing South African Windsnyer-type indigenous and Large White x Landrace pigs

Ingredient g/kg (as fed)	CON	LMC	HMC
Maize meal	667.8	526.1	396.6
Soyabean oilcake	200.0	184.1	173.7
Full fat soyabean cake	70.0	115.0	155.0
Ensiled maize cobs	0	100.0	200.0
Molasses	19.0	19.0	19.0
Sunflower oil	7.0	20.0	20.0
Limestone	6.0	5.0	4.0
Monocalcium phosphate	16.2	17.3	18.2
Chromium oxide	2	2	2
Salt	4.0	3.0	3.0
Lysine HCL	4.0	4.5	4.5
[b]Vitamin-Mineral Premix	4.0	4.0	4.0
	Laboratory Analyses g/kg DM		
Ether extract	40.0	42.0	54.4
Crude protein	180.2	186.4	193.8
Neutral detergent fibre	149.8	167.1	411.5
Acid detergent fibre	65.5	106.3	133.1
Gross energy MJ/kg	17.5	17.6	17.8

[b] Provided the following per kg of diet: 6500 IU vitamins A; 1200 IU D_3; 40 IU E; 2 mg K_3; 1−5 mg B_1; 4.5 mg B_2; 0.03 mg B_{12}; 2.5 mg B_6; 25 mg niacin; 12 mg calcium pantothenate; 190.5 mg choline; 0.6 mg folic acid; 0.05 mg biotin; 40 mg manganese; 100 mg zinc; 125 mg copper; 1 mg iodine; 100 mg ferrous; 0.3 mg selenium

4.2.2.2 Measurements

The pigs were weighed individually at the start of the trial and at the end of the trial. The pigs consumed feed freely. Feed intake was measured daily. Feed intake was measured by weighing the feed offered and subtracting the refusals. The preference of the test diets relative to the reference diet was calculated as the percentage contribution of the test diet to the total feed intake according to the following equation:

% Preference = 100 x (Intake of test diet/Total diet intake); Preference values ranged between 0 and 100%.

4.2.3 Experiment 2 Effect of diets containing ensiled maize cobs on nutrient digestibility and colon fermentation

4.2.3.1 Pigs, diets, housing and experimental design

Fifteen LW x LR and 15 SAWIP pigs, unbalanced on sex (3 males and 2 females per diet for each breed), that were part of a growth performance trial for eight weeks were used for the digestibility and colon fermentation study and weighed 70 ± 6.9 and 49 ± 8.1 kg respectively at the onset of the study. The two breeds stayed in similar pens described in Experiment 1. Five LW x LR and SAWIP pigs consumed one of

three diets; CON, LMC and HMC, in a random block design. Each pig received an allocation of feed daily in the morning based on their ability to finish with 10 % adjustments made on those that managed to finish their allocation. Feed intake determination involved weighing refusals every morning and subtracting them from feed offered. The diets were supplemented with 2 g chromium oxide (Cr_2O_3) marker per kg to determine digestibility coefficients of the nutrients. The pigs adapted to diets containing Cr_2O_3 for five days and thereafter, faecal collection took place for five days.

4.2.3.2 Sampling procedures

Representative feed samples from each diet were taken each time diets were mixed and stored at -20 °C for laboratory analyses. Faeces were collected by grab sampling on day 6 from 0800 to 1300h from each pig and stored at -20 °C for subsequent laboratory analyses. At the end of the collection period, the faeces were thawed overnight and dried at 60 °C for 24 hours before analysis. The 5-day faeces for each pig were combined and mixed after the drying period and then a representative sample analysed. Apparent total tract digestibility (ATTD) was calculated as:

ATTD = 100 – (100 x % Indicator feed / % Indicator faeces x % Nutrient faeces / % Nutrient feed)

At the end of the digestibility experiment the pigs were taken to the abattoir situated less than 1 km from the pens at around 0800 h. Pig processing followed routine abattoir procedures, which included an ante-mortem inspection and rest for the pigs before slaughter. The pigs were then stunned with an electrical stunner set at 220 V and 1.8 A with a current flow for 6 s and exsanguinated within 10 s of stunning. Dehairing and evisceration followed and the gastrointestinal tracts were set aside for sample collection. About 15 - 20 g digesta samples were obtained from the colon within 0.5-1 h after slaughter for volatile fatty acid (VFA) determination. A 10 cm section of the proximal colon 50 cm from the ileo-caecal junction was ligated, incised and its total contents collected for determination of VFA concentrations. The contents were put in 50 ml plastic bottles and frozen immediately at -20 °C pending analysis.

4.2.4 Laboratory analyses

4.2.4.1 Proximate and chemical analyses

The DM of the feed and faecal samples was determined by drying the samples at 100 ^{0}C for 24 hours according to the procedure of AOAC (1990). After drying, samples were ground through a 1 mm screen (Wiley mill, Standard Model 3, Arthur H. Thomas Co., Philadelphia, PA, USA) for chemical analyses. Following the procedures of Van Soest *et al*. (1991), the neutral detergent fibre (αNDF) concentration were determined using heat stable α-amylase (Sigma-Aldrich Co. LTD., Gillingham, UK, no. A-1278) with sodium

sulphite and the ADF concentration were determined using the Fibretec System equipment (Tecator LTD., Thornbury, Bristol, UK). Separate samples were used for ADF and αNDF analysis and both included residual ash. The nitrogen (N) content of the feed was determined with a N analyzer (FP-428, Leco Corp., St Joseph, MI) using a combustion method (990.03; AOAC, 1997). Crude protein was calculated by multiplying the N content by 6.25. Crude fat was measured using AOAC Soxhlet method (ID 960.39; AOAC, 1997). Ash (ID 942.05) and ether extract (EE, ID 963.15) were determined according to the procedure of AOAC (1990). The gross energy (GE) was determined with bomb calorimetry (MS-1000 modular calorimeter, Energy Instrumentation, Centurion, South Africa). Chromium was determined following the procedure described by Fenton and Fenton (1979).

4.2.4.2 Determination of colon digesta VFA concentrations

The colon digesta were stored frozen in sealed containers within 1 h of collection. After the digesta were thawed ~2 g was mixed in 8 mL purified water and centrifuged at 12 000 x g for fifteen minutes and about 2ml of the supernatant (digesta liquor) transferred into a test tube. A clean-up procedure modified from Siegfried *et al*. (1984) was then used to deproteinize the colon digesta liquor samples as well as removing the sugars. Briefly, a 1.5 ml sample of digesta liquor was transferred into a 1.7 ml centrifuge tube and centrifuged at ~12 000 g for 10 minutes. Then 600 µl of supernatant was transferred to duplicate empty 1.7 ml centrifuge tubes. After centrifugation, 600 µl of calcium hydroxide solution (CHS; 52.9 g Ca $(OH)_2$ homogenized in 200 ml pure water) and 300 µl of cupric sulphate solution (CSR; a solution of 50.0 g of $CuSO_4.5H_20$ and 2.0 g of crotonic acid (2-butenoic 38-acid, Sigma nr. C4630) diluted to volume (500 ml) with ultra-pure water) were added to the tubes. The tubes were then capped, vortexed and frozen. The tubes were thawed, centrifuged at 12 000 x g for 10 minutes, and 1 000 µl of supernatant transferred to clean 1.7 ml centrifuge tubes containing 28 µl of concentrated H_2SO_4 which were then capped and frozen. The tubes were again allowed to thaw after which they were frozen again. The tubes were again thawed and centrifuged for 10 minutes. The supernatant was transferred to the vials used for High Performance Liquid Chromatography (HPLC). The vials were capped and stored in a refrigerator. The HPLC was performed using a Waters 717 Autosampler (Empower 2 software) equipped with a Waters 2487 Dual λ Absorbance Detector and a Biorad Aminex HPX 87H (65 °C) column. The mobile phase was composed of 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. The injection volume was 30 ul and UV detection was done at 220 nm. Standard solutions containing reagent grade acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acids in various proportions were also prepared for chromatography and the widths of the half peak-heights and the peak-heights of these solutions were used to calculate the unknown concentrations of VFA in the samples.

4.2.5 Statistical analyses

The preference, ATTD and VFA concentrations for diets containing ensiled maize cobs was analysed according to a factorial arrangement of treatments with two breeds (SAWIP and LW x LR) and three diets (CON, LMC, HMC) using the GLM procedure of the statistical package of SAS (SAS 9.3, Inst. Inc., Cary,

NC). All data were tested for normality and homogeneity and comparisons were made to the 95 % significance level. Metabolic weight (Initial $Wt^{0.75}$) was used as a covariate for the digestibility data. Preference data were square root transformed, ATTD coefficients of ADF and EE were square transformed while the colon digesta VFA concentrations were all log transformed except for proportion of VA which was inverse transformed to achieve normality. The following model was used:

$$Y_{ijk}=\mu+D_i+B_j+(D*B)_{ij}+e_{ijk}$$

Where Y_{ijk} is the percentage preference, ATTD coefficient of a nutrient or VFA concentration for the diet observation (i), and the breed (j); μ is the mean of all observations; D_i is the diet (CON, LMC, HMC); B_j is the breed effect (SAWIP, LW x LR). The interaction between diet and breed was denoted by $(D*B)_{ik}$ and e_{ijk} is the unexplained random error. In addition, the average preference for each diet in each breed was compared with the 50 % no-effect level by Student's t-tests. A value of 50 % would indicate an indifference respect to the reference diet, whereas values significantly higher or lower than 50 % would indicate a significant preference or aversion respectively. Pearson correlation coefficients between feed preference and ATTD nutrient digestibility per breed and per diet were obtained by using the CORR procedure of SAS. The Bonferroni method and the PDIFF statistic of SAS (SAS 9.3 Inst. Inc., Cary, NC) were used to separate the means.

4.3 Results

The preference values for diets containing low (LMC) and high (HMC) ensiled maize cobs inclusion levels offered to SAWIP and LW x LR pigs in a double-choice set-up are shown in Table 4.3. There were breed by diet interactions ($P < 0.05$) in period 1 (P1; 0-3 days) only. Diet influenced preference values in P1 ($P < 0.05$). Overall, the SAWIP's preference for the CON was greater than for the HMC diet ($P < 0.05$). The two breeds' preference values for the LMC and HMC diets were lower ($P < 0.05$) than the 50 % preference index in P1. In period 2 (P2), the LW x LR preference values for LMC and HMC diets were still lower ($P < 0.05$) than the 50 % preference index while the SAWIP's were not.

Table 4.3 [1]Preference values for control (CON) anddiets containing ensiled maize cobs at low (LMC) and high (HMC) inclusion levels offered to South African Windsyner indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) crosses in a double-choice set-up

	SAWIP			LW x LR				P-Values		
Nutrient	CON	LMC	HMC	CON	LMC	HMC	SD	Breed	Diet	BreedxDiet
Period 1	58.6^{b}	32.7^{a†}	33.0^{a†}	41.6^{ab}	44.2^{b†}	41.9^{b†}	14.0	0.254	0.042	0.019
Period 2	40.1	39.4	37.2	39.5	32.0†	34.1†	20.0	0.543	0.713	0.761
Overall	49.4^{b}	37.2^{ab†}	34.2^{a†}	41.7^{ab†}	38.3^{ab†}	38.0^{ab†}	14.6	0.860	0.137	0.521

[1]Preference is expressed as the intake of the test diet as a percentage of the total intake. [a,b] Means with different letters in a row differ significantly (P<0.05); [†]Values with this superscript are different from the neutral value of 50 (P < 0.05). A value of 50 indicates that the diet under study and the reference diet are equally preferred.SD – Standard deviation

The ATTD coefficients of nutrients in the CON, LMC and HMC diets fed to SAWIP and LW x LR pigs are in Table 4.4. The ATTD coefficients of CP, ADF, Hemi and NDF in the HMC were greater (P < 0.05) than in the CON. The SAWIP had greater (P < 0.05) NDF digestibility coefficients than the LW x LR. There was no correlation (P > 0.05) between preference and digestibility coefficients of DM, OM, CP, GE, ADF, NDF and EE for the SAWIP (results not shown).

Table 4.4 Apparent total tract digestibility (ATTD) coefficients of nutrients in control (CON) and diets containing ensiled maize cobs at low (LMC) and high (HMC) inclusion levels fed to South African Windsnyer-type Indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) pigs

	SAWIP			LW x LR				P-Values		
Nutrient	CON	LMC	HMC	CON	LMC	HMC	SD	Breed	Diet	BreedxDiet
DM	0.81	0.76	0.76	0.78	0.76	0.76	0.030	0.473	0.152	0.715
OM	0.83	0.79	0.79	0.80	0.79	0.78	0.030	0.275	0.163	0.717
CP	0.75^{ab}	0.78^{b}	0.80^{b}	0.70^{a}	0.77^{b}	0.80^{b}	0.037	0.132	<0.0001	0.333
NDF	0.67^{abc}	0.69^{bc}	0.77^{d}	0.59^{a}	0.64^{ab}	0.74^{cd}	0.053	0.038	0.002	0.733
ADF	0.37^{a}	0.47^{ab}	0.68^{b}	0.36^{a}	0.41^{a}	0.65^{b}	0.100	0.597	0.009	0.899
Hemi	0.68^{ab}	0.71^{b}	0.77^{b}	0.60^{a}	0.68^{ab}	0.75^{b}	0.053	0.109	0.004	0.621

[a,b] Means with different letters in a row differ significantly (P<0.05)
DM - dry matter; OM – organic matter; CP – crude protein; NDF- neutral detergent fibre; ADF - acid detergent fibre; Hemi – hemicellulose (calculated as the difference between NDF and ADF)

The effect of diets containing low (LMC) and high (HMC) ensiled maize cob levels on concentrations and molar proportions of volatile fatty acids in the colon of South African Windsnyer-type indigenous pigs (SAWIP) and Large White x Landrace crosses (LW x LR) are shown in Table 4.5. There were breed x diet interactions (P < 0.05) for proportions of isobutyric acid (IBA) and butyric acid (BA), IBA concentration and acetic to butyric acid (AA:BA) and BA:IBA ratios. There were breed differences (P < 0.05) in concentrations

of total VFA, AA, PA and BA and molar proportions of AA, IBA and BA. Dietary effects ($P < 0.05$) were observed for total VFA, AA, PA, BA and VA concentrations.

Table 4.5 The effect of diets containing ensiled maize cobs at low (LMC) and high (HMC) levels on concentrations and molar proportions of volatile fatty acids in the colon of South African Windsnyer-type indigenous pigs (SAWIP) and Large White x Landrace crosses (LW x LR)

Breed	SAWIP			LW x LR				P-Values		
	CON	LMC	HMC	CON	LMC	HMC	SD	Diet	Breed	Breed x Diet
Total VFA mmol/L	221^{bcd}	219^{bc}	178^{a}	249^{cd}	252^{d}	215^{b}	25.3	0.0002	0.001	0.700
Molar proportions										
AA	0.65^{c}	0.65^{bc}	0.66^{c}	0.62^{ab}	0.60^{a}	0.65^{c}	0.026	0.054	0.010	0.171
PA	0.24	0.24	0.25	0.24	0.25	0.24	0.028	0.892	0.927	0.596
BA	0.07^{a}	0.08^{a}	0.09^{ab}	0.11^{c}	0.10^{bc}	0.09^{ab}	0.019	0.623	0.004	0.054
VA	0.02^{ab}	0.02^{a}	0.01^{ab}	0.02^{a}	0.02^{ab}	0.01^{b}	0.005	0.062	0.346	0.980
IBA	0.02^{b}	0.02^{b}	0.01^{b}	0.009^{a}	0.01^{b}	0.01^{b}	0.003	0.659	0.001	0.031
VFA mmol/L										
AA	144^{b}	141^{b}	112^{a}	154^{b}	152^{b}	139^{b}	14.9	0.001	0.007	0.134
PA	53^{abc}	53^{ab}	44^{a}	59^{bc}	64^{c}	52^{ab}	9.4	0.018	0.019	0.870
BA	16^{a}	18^{a}	16^{a}	29^{b}	25^{b}	19^{a}	5.4	0.051	0.0008	0.303
VA	4.2^{c}	3.6^{bc}	2.3^{a}	4.7^{c}	3.8^{c}	2.6^{ab}	1.35	0.003	0.547	0.984
IBA	3.7^{c}	3.3^{c}	2.4^{ab}	2.3^{a}	3.1^{bc}	2.7^{abc}	0.68	0.071	0.135	0.036
AA:PA	2.7	2.8	2.6	2.6	2.4	2.7	0.40	0.922	0.508	0.221
AA:BA	9.4^{b}	8.0^{b}	7.8^{b}	5.5^{a}	6.0^{a}	7.7^{b}	1.53	0.459	0.002	0.038
BA:IBA	4.4^{a}	6.0^{a}	6.6^{ab}	14.2^{c}	8.4^{b}	6.8^{ab}	2.05	0.546	<0.0001	0.002

^{a,b} Values with different letters in a row differ ($P<0.05$); SD – standard deviation; AA - acetic acid; PA - propionic acid; BA - butyric acid; VA - valeric acid; IBA - isobutyric acid

4.4 Discussion

The pigs in the current study were evaluated over two periods of 3 days, which were deemed sufficient to determine preferences. Sola-Oriol *et al*. (2009a) and Seabolt *et al*. (2010) observed that differences in preferences can be detected after 1 to 2 days in weaners and these differences persist for longer periods. Although the response of growers is not known, it was assumed to follow the same pattern as that of weaners. Sola-Oriol *et al*. (2009a) observed that there were no differences in preference patterns between weaners and post weaners. Control comparisons acted as indicators of the presence of confounding factors like placement of feeders, temperature and ventilation variations within the room (Sola-Oriol *et al*., 2009a; Seabolt *et al*., 2010). Differences in dietary preferences were only recorded in the first period despite the fact that the diets differed in fibre levels. Sola-Oriol *et al*. (2009b) attributed differences in preferences between hulled and dehulled oats, and unhulled short grain rice and polished white rice to differences in fibre levels. Fibre affects preference of feeds through difficulty in chewing and increased bulkiness of diets, which are magnified in weaners compared to older pigs (Whittemore *et al*., 2001; Sola-Oriol *et al*., 2009b; Seabolt *et al*., 2010). Ndou *et al*. (2013) however explained similar feed intake in diets containing maize cobs at 80,

160, 240 g/kg levels by uniformity of water holding capacity and bulk densities of the maize cob based feeds. Results from the current study indicate that pigs quickly adapted to the maize cob diets and were not influenced by the negative effects often associated with fibrous ingredients. Another possible explanation is that ensiling changed the nature and texture of the maize cobs and improved their preference. Ensiling the maize cobs was expected to positively influence the diet odour and texture through the various volatile acids produced during fermentation and the reduction in dry matter and NSP levels, thus improving preference. The positive benefit of the maize cobs fermentation should however be studied further by comparing them with non-fermented maize cobs.

The observation that the SAWIP had a higher preference on the CON-CON combination in the first period and overall compared to the LW x LR was unexpected given the assertion that indigenous pigs digest and utilize high fibre diets better than the LW x LR crosses (Kanengoni *et al*., 2002; Ndindana *et al*., 2002). However in line with the above mentioned assertion, the LW x LR consistently then showed a preference for the CON diets compared to the LMC and HMC diets in the second period as well unlike the SAWIP where the preference for the CON was only in the first period. The SAWIP preferences for the LMC and HMC diets improved by 6.7 and 4.2 % respectively while that of the LW x LR reduced by 12.2 and 7.8 % respectively in the second period. This can be interpreted to say that SAWIP have a better preference for diets containing ensiled maize cobs than the LW x LR crosses and that they adapted quickly to the diets leading to improved consumption of the diets containing maize cobs in the second period. It could be that the LW x LR's preferences for the CON diet were influenced by the desire to meet their energy needs. Sola-Oriol *et al*. (2009a) attributed a negative correlation between crude fibre and preference to a low energy density of high fibre diets. Although the indigenous pigs of Southern Africa have been reported to survive on fibrous feeds, no studies have been done to investigate whether they actually have a preference for these diets compared to the commercial breeds. This study demonstrates that this could be the case but further investigations are required.

Apparent digestibility of CP, NDF, Hemi and ADF improved as ensiled maize cob level increased in this study contrary to previous findings (Stanogias & Pearce 1985; Kanengoni *et al*., 2002; Ndubuisi *et al*., 2008). This can be partly explained by the fact that the pigs were well adapted to the diets after 6 weeks. Kyriazakis (2011) also reported that the capacity of pigs' caecum and colon to ferment fibre can increase at high fibre levels. In addition to adaptation and increased substrate for fermentation in the colon with increased fibre levels, the improvement in digestibility can also be attributed to ensiling. This is corroborated by Jørgensen *et al*. (2010) who reported improvements in ileal digestibility of DM, OM and energy by 6 and 3 percentage units after fermentation of barley and wheat respectively, whereas the total tract digestibility was increased by 2 to 3 and 1 to 2 percentage units, respectively. Similarly, Lyberg *et al*. (2006) found an increase in OM digestibility of 9 % units at the ileal level and 2 % for the total tract in pigs fed fermented barley/wheat based diets compared to the same fed as dry feed. Jørgensen *et al*. (2010) then suggested that fermenting ingredients, especially those with low digestibility and fermentability, can be a strategy to improve their energy value for pigs. The observation that ATTD of NDF in SAWIP was higher than in the LW x LR is consistent with the results from Kanengoni *et al*. (2002). In the Mukota pigs, digestibility of NDF decreased

by 16 % while in the LW x LR it decreased by 21 % when the inclusion level of maize cobs in the diet was increased from 100 to 300 g/kg (Kanengoni *et al*., 2002). Explanations for the breeds' differences in digestibility of nutrients in high fibre diets are that gut capacity and intestinal microbial populations differ among age groups and breeds (Kyriazakis & Emmans, 1995; Whittemore *et al*., 2003; Thacker & Haq, 2009). Urriola & Stein (2012) suggested that the superiority of the Meishan pig (an indigenous Chinese pig) to digest insoluble fibre over the Yorkshire could be a result of differences in the intestinal microbial populations or types. One indicator of the extent of fermentation is the quantity and quality of fermentation products produced.

Even though the SAWIP had higher ATTD of ADF and NDF than the LW x LR, their total colon VFA concentration was lower. The proportional contribution of AA and IBA to the total VFA concentration was higher in the SAWIP than in the LW x LR indicative of more efficient carbohydrate and higher protein fermentation. Bindelle *et al*. (2008) reported that the proportion of acetate is reciprocal to the concentration of butyrate due to bacterial cross-feeding as butyrate-producing bacteria can be net utilizers of acetate but this was not reflected in this study. Macfarlane & Macfarlane (2003) proposed different fermentation pathways with acetate arising from pectin and xylan breakdown, and acetate and propionate being produced from arabinogalactan and butyrate only formed in substantial amounts from starch. This would be consistent with the contributions from maize cobs to the diet. Maize cobs are high in xylan and low in starch (Ebringerová & Heinze, 2000; Božović *et al*., 2004; Vázquez *et al*., 2006). The results raise the question of whether there are differences in efficiency of absorption of the VFAs between the LW x LR and the SAWIP. It could also be that the total VFA concentration was affected more by the volume of digesta fermented than the efficiency of fermentation, of which the former was higher in the LW x LR while the latter was higher in the SAWIP.

There were marked differences in how the two breeds responded to the diets with regard to proportions of IBA and BA production. Woldeghebriel *et al*. (2012) obtained BA:IBA ratios of 5:1, 9:1, 6:1 and 15:1 in the different diets while in this study they ranged from 4:1, 6:1, 7:1 in the SAWIP and 14:1, 8:1, 7:1 in the LW x LR for the CON, LMC and HMC diets respectively. The SAWIP had higher levels of IBA than BA as indicated by the lower ratios. Higher levels of IBA are indicative of extensive protein degradation, and this would mean that relatively higher quantities of protein were available post-ileum in the SAWIP. Isobutyrate is also considered toxic to the colon mucosa (Montagne *et al*., 2012) and its reduction as the levels of fibre increased is probably indicative of greater fermentative capacity. Hermes *et al*. (2009) reported that pigs fed diets containing 20 % protein showed higher concentration of products of protein fermentation in the colon while a simultaneous increase of fibre to the diet not only increased fermentation of carbohydrates, but also decreased concentration of products of protein fermentation. The results support the premise that indigenous pig breeds require less protein in dietary formulations than the commercial breeds. However, increased amino acid catabolism as an explanation for the differences seen between the breeds in this study needs to be taken with caution because the production of isovaleric acid, which is also a product of protein degradation, was very small and comparable between the breeds. Valeric acid levels increase when there is catabolism of branched chain amino acids especially isoleucine (Van Nevel *et al*., 2006) and there were no differences between the breeds in the study. Butyric acid is believed to be clinically important because of its

metabolic role in the health of the colonic mucosa and use as a source of energy by colonocytes (Roediger 1980; Shi & Noblet, 1993), so the results would indicate that the LW x LR had a healthier intestinal environment. The fermentation patterns could also have been influenced by the presence of different types of microorganism in the two breeds.

4.5 Conclusions

Ensiled maize cobs added to pig diets at 200 g/kg improved digestibility of nutrients. Although the SAWIP demonstrated a preference for the control diet, they adapted quicker to diets containing ensiled maize cobs than the LW x LR. More research to determine to what extent the preference was influenced by nutrient level such as energy or protein concentration or alternatively to sensorial regulation is proposed. The SAWIP digested nutrients better than the LW x LR in the high fibre diets. This could be indicative of a breed influence on diet preference.

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Chapter 5

5 *Genomic analysis of faecal microbial populations of South African Windsnyer-type indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) crosses fed diets containing ensiled maize cobs*

Abstract

The composition of faecal bacterial communities in SAWIP and LW x LR crosses was compared using metagenomic pyrosequencing of 16S rRNA genes to explain the differences in fibre utilization between these breeds. Eight LW x LR and five SAWIP pigs were evaluated on a diet without maize cobs (CON) and a diet containing 200 g/kg ensiled maize cobs (HMC) in a completely randomized block design. The rarefaction estimates showed that there were no differences in microbial diversity between the breeds and diets after 8 weeks. The breed x diet plots showed differences between the SAWIP and LW x LR on the CON diet. Dominant phyla detected were *Firmicutes* (67.1%), *Bacteroidetes* (15.2%), and *Spirochaetes* (13.6%) which are all involved in cellulolytic digestion. There were no differences in Operational Taxonomic Units (OTU's) between the breeds at the phylum level. At the class level, the ratios of *Bacteroidia* to *Clostridia* in SAWIP on the CON and HMC diets were similar (0.37 vs 0.39); and were different (0.24 vs 0.1) in LW x LR implying breed differences in microbial responses to diets with and without ensiled maize cobs. *Verrucomicrobiae,* was detected in SAWIP and LW x LR on HMC diet and not on the CON diet. There was a breed x diet interaction ($P < 0.05$) for *Oscillospira*. *Oscillospira* increased in LW x LR as diet changed from CON to HMC while it decreased in the SAWIP. Analysis of the microbiome revealed differences occurring between the two breeds but not between the diets. There is need to define the relationship between the faecal microbiome and pigs' efficiency in digesting fibrous diets.

Key words: fibre digestion, fermentation, microbiome

5.1 Introduction

The mechanisms through which South African WIndsnyer-type indigenous pigs (SAWIP) digest NDF better than the crosses of Large White and Landraces (LW x LR) as reported in Chapter 4 are not fully understood. Similar observations were made with regard to other indigenous pigs of Southern Africa compared to Large White crosses (Ndindana *et al*., 2002; Kanengoni *et al*., 2004). Resident gastrointestinal tract microflora may play key roles in these breeds. The diverse microbial communities colonizing the pig gastrointestinal tract are dependent on age, diet composition, rearing environment and genotype. In humans, diet and lifestyle modify gut microflora (O'Flaherty & Klaenhammer, 2010; Kau *et al*., 2011; Gravitz, 2012). By far, the strongest determinant of the gut microbial diversity is the host diet (Apajalahti *et al*., 2001, 2004; Oviedo-Rondón *et al*., 2006) where any change in feed composition not only affects the species of microbes in the gut, but the type and abundance of fermentation products. This therefore directly influences gut health and nutrient availability.

The pig industry all over the world faces the challenge of effectively utilizing readily available fibrous feeds from the agro-processing industries. While recent research (Montagne *et al*., 2012; Urriola & Stein, 2012) focused on impact of the fibrous sources on growth performance, there is a need to quantify potential beneficial effects of high fibre diets on intestinal health and welfare. In South Africa, for example maize cobs are a readily available feed ingredient, which ought to be exploited more in pig diets. Studies on the potential of maize cobs as a feed ingredient focused on the growth performances of pigs fed low inclusion levels comparable to those fed conventional fibrous sources (*et al* Ndindana *et al*., 2002; Kanengoni *et al*., 2004; Mashatise *et al*., 2005). There is a need to investigate how maize cobs influence microbial communities and intestinal health in grower pigs.

Culture dependent methods are inadequate to elucidate microbial profiles in the hindgut since only 1 % of microbial communities are culturable (Hugenholtz *et al*., 1998). Molecular techniques can unravel complex intestinal microbial communities in humans and animals (Van der Wielen *et al*., 2002; O'Flaherty & Klaenhammer, 2010; Kau *et al*., 2011; Gravitz, 2012). The objective of this study was to evaluate the influence of breed and diet on the faecal microbiome in commercial and indigenous South African breeds fed diets containing ensiled maize cobs using meta-genomic pyrosequencing of the 16S rDNA genes.

5.2 Materials and Methods

5.2.1 Diets

Diets with inclusion levels of 0 and 200 g maize cob/kg of diet (as fed) were formulated to provide 14 MJ/kg digestible energy (DE), 180 g crude protein (CP)/kg and 11.6 g lysine /kg which meet and exceed the requirements of growing pigs (NRC, 1998) (Table 4.2). This resulted in two treatments namely; control diet without maize cobs (CON), and diet containing 200 g ensiled maize cob/kg (HMC). The silage drums were

opened weekly when mixing the diets to minimize spoilage. The research was approved by the Animal Ethics Committee of the Agricultural Research Council, Animal Production Institute (ARC-API).

5.2.2 Pigs, housing and experimental design

Large White × Landrace crossbred pigs (LW x LR) (n = 25) weighing 25 ± 5 kg body weight and 15 South African Windsnyer-type Indigenous pigs (SAWIP) (n = 15) weighing 15 ± 4 kg were randomly selected from the ARC-Irene pig breeding units and evaluated in a growth performance study. As the SAWIP and LW x LR differ in their mature body weight (300 – 350 kg vs 140-180 kg), growing pigs of a similar degree of maturity (0.10 of adult body weight) were chosen from each breed for this study. The LW x LR's pens measured 2 x 1.5 m and the SAWIP's, 1.5 × 0.9 m, and were in environmentally controlled houses with the temperature ranging from 22 – 25 °C. The pigs consumed one of two diets; CON and HMC as described in 5.2.1 in a completely randomized block design. Eight LW x LR and five SAWIP pigs were evaluated per diet for the growth performance study but comparisons for faecal microbe profiles were done on three male pigs per breed per treatment. Pigs' daily allocation of feed came in the morning based on their ability to finish and 10 % adjustments were made on those that managed to finish their daily allocation. Daily feed intake was calculated from feed offered less the refusals, which were weighed every morning. The feeders were checked and adjusted twice each day to ensure constant access to fresh feed and minimize any possible wastage. The pigs were blocked by weight and breed when assigned to the treatments. Water was freely available through nipple drinkers. The number of days to slaughter, from study inception was 56 days for the LW x LR and 75 days for the SAWIP. At the end of the growth, study the pigs were taken to the abattoir situated less than 1 km from the pens at around 08.00 h and slaughtered.

5.2.3 Sampling procedures

The pigs were processed according to the routine abattoir procedures, which included an ante-mortem inspection and rest for an hour for the pigs before slaughter. Prior to slaughter, the pigs were electrically stunned with an electrical stunner set at 220 V and 1.8 A with a current flow for 6 s and exsanguinated within 10 s of stunning. The pig carcasses were dehaired by scraper and knife by hand following scalding at 63 °C and the remaining hairs were removed with a gas naked flame. Evisceration followed and the gastrointestinal tracts were set aside for sample collection. For microbial profiling, faecal samples were obtained aseptically from the rectum and kept at -20 °C. Care was taken not to cross-contaminate samples by cleaning dissection instruments with 70 % ethanol and changing gloves between dissections.

5.2.4 Laboratory analyses

5.2.4.1 DNA extraction and PCR amplification of the 16S gene

The faecal samples were thawed and 0.25 g of each sample was put into 1.5 ml sterile microtubes. Genomic Deoxyribonucleic Acid (DNA) was extracted from the faecal samples using the PowerSoil®DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA), following the procedure provided by the manufacturer. During faecal DNA isolation, poor DNA concentrations were obtained due to different compounds present in the faecal material. The process was therefore optimised by repeating the procedure resulting in good DNA concentrations before proceeding to downstream applications.The DNA was quantified using the Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA., USA). The V3 and V4 regions of the 16S rDNA gene was amplified on a GeneAmp® Polymersae Chain Recation (PCR) System 9700 (Applied Biosystems, Foster City, California, USA) using modified 16S primer pairs S-D-Bact-0341-b-S-17 (3'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-5') and S-D-Bact-0785-a-A-21 (3'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-5') with adapter overhang which are complementary to Illumina forward, reverse, and multiplex sequencing primers (Klindworth *et al*., 2013, 16S Sample Preparation Guide 15044223-Illumina). The PCR reaction (25 µl) mixtures were prepared using 0.5 µl of the genomic DNA template (20 ng/UL), 12.5 µl of DreamTaq Green, PCR master Mix (Thermo Scientific, Waltham, Massachusetts, USA), 0.5 µl of each primer (10 mM) and 11 µl water. A touchdown PCR method (Don *et al*., 1991) was performed with the following modifications: an initial denaturation of the genomic DNA at 95°C for 3 min, followed by six cycles consisting of denaturation at 95°C for 30 sec, annealing temperature stepdown every second cycle of 1°C (70°C to 65°C); an extension step at 72°C for 30 sec; followed by another 30 cycles consisting of denaturation at 95°C for 15 sec, annealing temperature at 65°C for 15 sec, an extension step at 72°C for 30 sec, and a final extension step at 72°C for 7 min. A 5 µl aliquot of the PCR products was separated on a 1% agarose gel and visualized under UV light. The PCR products were purified using Qiaquick PCR purification kit (250) (Qiagen), following the manufacturer's instructions. The concentrations of the purified PCR products were determined using a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, Ca. USA).

5.2.5 Library preparation and Illumina sequencing

Purified PCR products were used as templates to perform an index PCR. This step consisted of attaching dual indices and Illumina sequencing adapters to each sample using the Nextera XT Kit. The protocol for index PCR was done according to the procedure provided by Illumina (16S Sample Preparation Guide, 15044223-Illumina). The PCR products were purified after indexing and multiplexed following the procedures prescribed by Illumina (16S Sample Preparation Guide, 15044223-Illumina). The library was sequenced using the Miseq version 2 platform (Illumina, Sand Diego, California, USA) with 300 by 300bp paired-end V3 reagent chemistry (Illumina MS-102-3003) at the Agricultural Research Council Biotechnology Platform (ARC-BTP).

5.2.6 Sequence processing and analysis

A combination of denovo and reference based operational taxonomic units (OTU) identification was carried out using the open_reference calling method implemented within Quantitative Insights Into Microbial Ecology (QIIME) software package. A default similarity level of 97 % was used to cluster sequences into individual OTUs and a single representative sequence from each clustered OTU was used to align to the Greengenes database (version: gg_13_5) (Caporaso *et al*., 2010). Taxonomic classification for each OTU was determined with Ribosomal Database Project (RDP) Classifier using a minimum confidence cutoff of 0.8. The OTUs with fewer than 100 sequences across all samples were excluded from further analysis. Estimates of distance matrices for both alpha and beta diversity calculation and a per-sample summary of OTU representation at various taxonomic levels were also calculated.

5.3 Statistical analysis

Operational taxonomic units with at least 100 sequences per OTU (n= 835) were identified in microbial communities from LW x LR and SAWIP pigs fed a control diet and a diet containing high maize cob levels. These were classified into two kingdoms, Bacteria (99.3 %) and Archea (0.7 %). A total of 99.3 % OTU's from the LW x LR on the CON diet, 99.6 % OTU's from LW x LR HMC, 98.85 % OTU's from SAWIP CON and 99.74 % OTU's from SAWIP HMC diets were assigned to the phylum level. The similarities and dissimilarities between the groups were evaluated by unweighted (based on presence or absence of taxa) and weighted (based on relative abundance) UniFrac based principal coordinates analysis. Abundant species were defined at an empirical cutoff of percentage abundance >1 %. Comparisons were made to the 95 % significance level. The Bonferroni and false discovery rate (FDR) procedures were used to correct for multiple test *P*-values.

5.4 Results

5.4.1 Species diversity and richness

Rarefaction curves using Phylogenetic Diversity (PD) and observed species metrics in the two breeds and diets and their interactions showed the extent of species diversity and richness. The shape of the curves obtained were similar in all samples and indicated a trend of diminishing chance of finding new phylotypes as sampling continued as shown in Figure 5.1. Rarefaction estimates showed that there were no differences in microbial diversity between the breeds and diets (Fig 5.1A and B). The breed x diet plots (Fig 5.1C) however showed significant differences between microbial communities in the SAWIP and LW x LR pigs on the control diet but not HMC diet. The individual diversity variations between the pigs suggest some genotypic influences (Fig 5.1D).

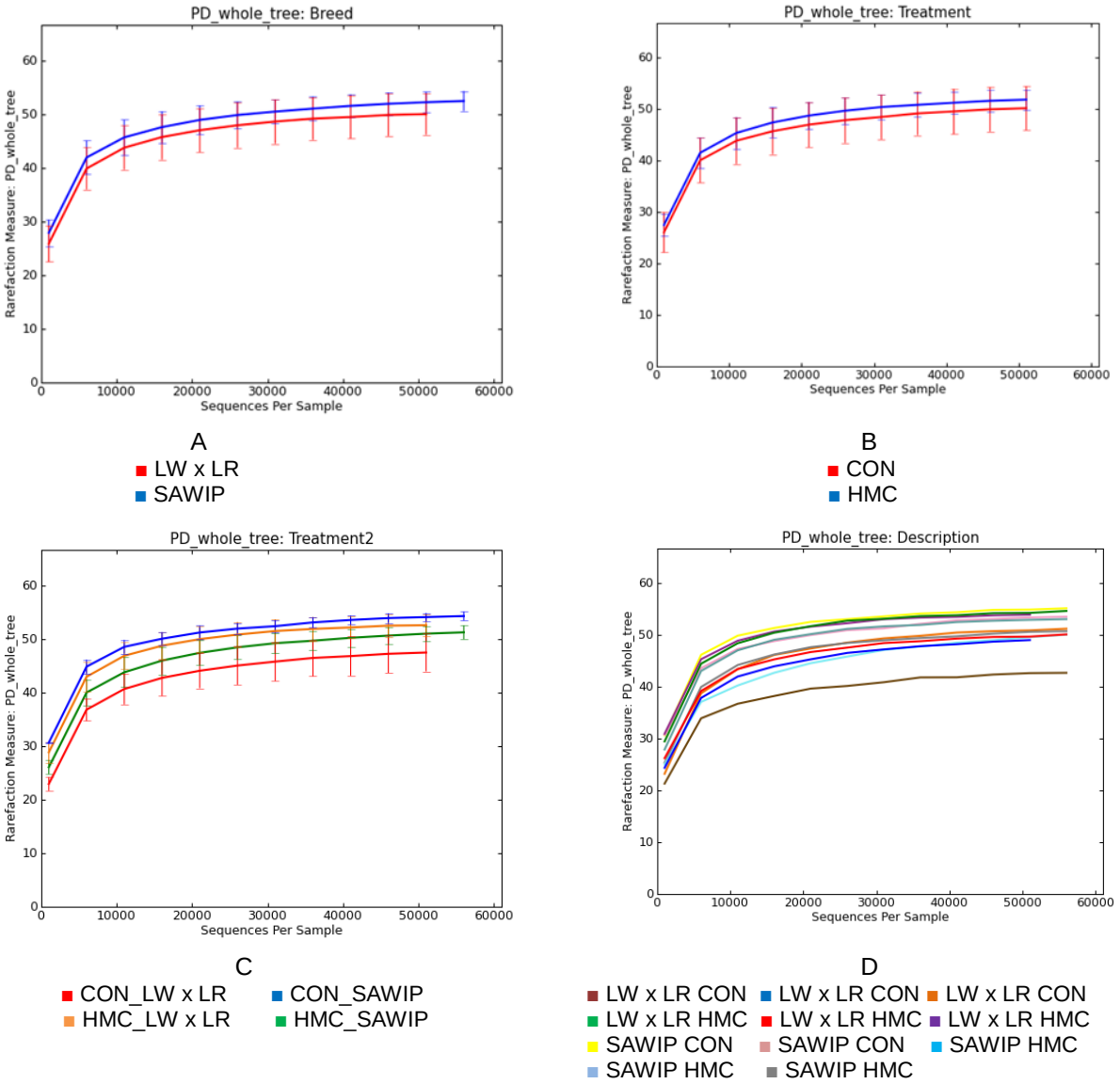

Figure 5.1 Rarefaction curves using Phylogenetic Diversity (PD) and observed species metrics in the Large White × Landrace crossbred pigs (LW x LR) and South African Windsnyer-type Indigenous pigs (SAWIP) pigs fed control (CON) and high maize cob level (HMC) diets for breeds (A), diets (B), breed x diet (C) and indiviudals (D)

Figures 5.2A and B show the unweighted UniFract analysis coloured to distinguish communities based on individual pigs, breeds and diet groups. Weighted UniFract analysis of the same data is shown in Figures 5.2 C and D. In both cases the data emphasized that there are individual pig differences and clustering of the gut microbial communities occurred based on breed (SAWIP; LW x LR) but not on diet (CON, HMC). Single outliers that did not cluster well with the other samples occurred in both the LW x LR and SAWIP breeds.

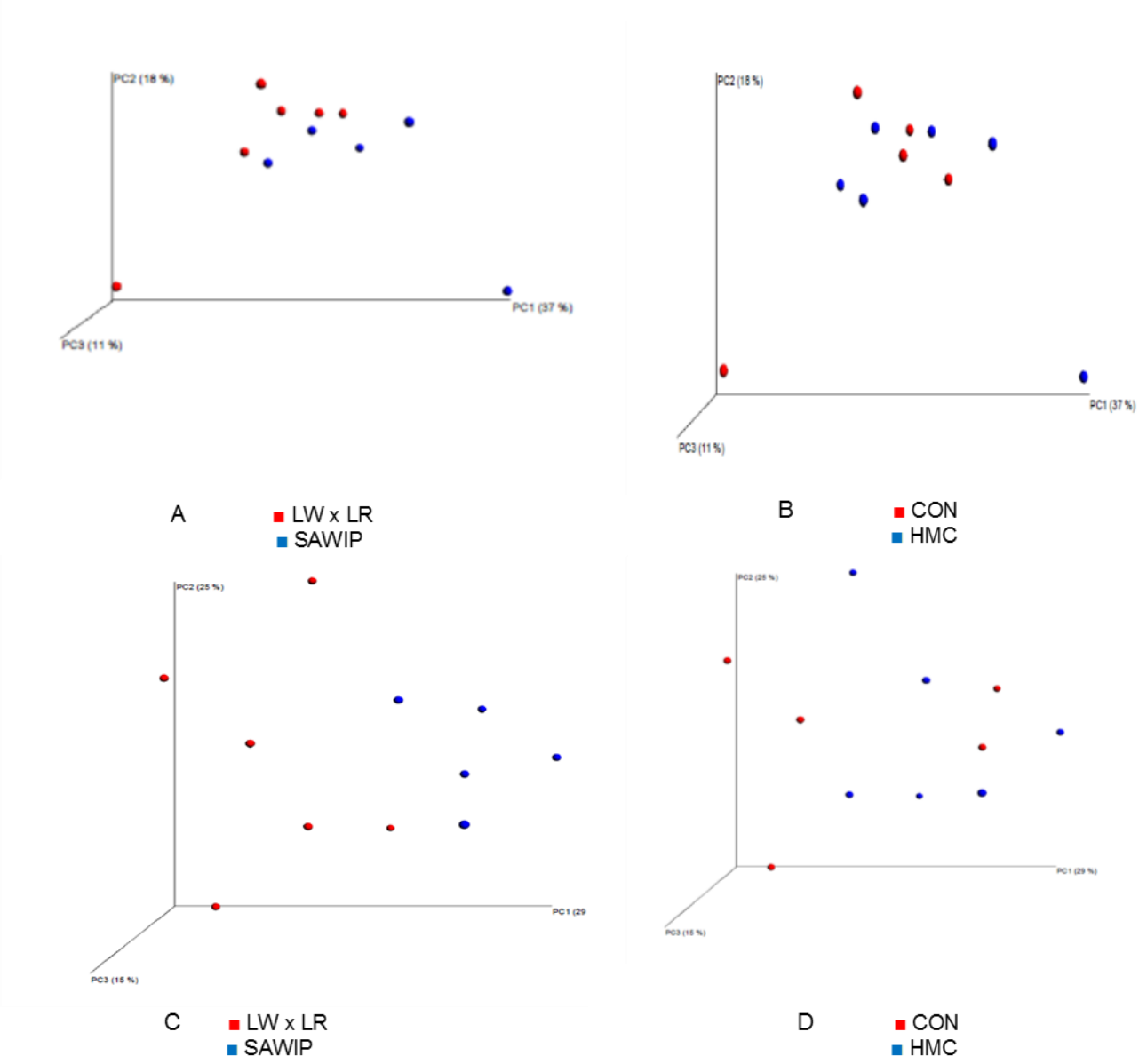

Figure 5.2 Unweighted UniFrac based principal coordinates plots of; A) the Large White × Landrace crossbred pigs (LW x LR) and South African Windsnyer-type Indigenous pigs (SAWIP) pigs; B) control (CON) and high maize cob level (HMC) diets; and weighted principal coordinates plots for C) the the LW x LR and SAWIP breeds and D) the CON and HMC diets

The OTU's were classified taxonomically into 14 phyla, 20 classes, 22 orders, 38 families and 41 genera as shown in Table 5.1.The following genera were present in the LW x LR and absent in the SAWIP; *Butrivibrio, Faecalibacterium, Desulfovibrio*, unidentified *Enterobacteriaceae*. Absent from the LW x LR and present in the SAWIP were *Bacteroides*, unidentified *RF16*, *Succiniclasticum, Peptococcus*, unidentified *Dethiosulfovibrionaceae*, unidentified *Cerasicoccaceae*, and *Akkermansia.*

Table 5.1 Taxonomic classification and proportions (%) of Operational Taxonomic Units (OTU's) from Large White × Landrace crossbred pigs (LW x LR) and South African Windsnyer-type Indigenous pigs (SAWIP) pigs

Phylum	Class	Order	Family	Genus	LW x LR (%)	SAWIP (%)
Actinobacteria (0.1 %)	*Coriobacteriia (0.1 %)*	*Coriobacteriales (0.1 %)*	*Coriobacteriaceae*	-	0.10	0.04
				Collinsella	0.05	0.02
Bacteroidetes (14.8 %)	*Bacteroidia (14.8 %)*	*Bacteroidales (14.8 %)*	*Prevotellaceae*	*Prevotella*	2.37	2.08
			Paraprevotellaceae	*CF231*	0.23	0.18
				Prevotella	0.42	0.18
				YRC22	1.10	1.50
			BS11	-	0.22	0.66
			Bacteroidaceae	*Bacteroides*	0.00	0.08
			RF16	-	0.00	0.02
			Porphyromonadaceae	*Parabacteroides*	0.12	3.94
			p-2534-18B5	-	0.15	0.40
			S24-7	-	5.10	8.74
Cyanobacteria (0.1 %)	*4C0d-2 (0.1 %)*	*YS2 (0.1 %)*	-	-	0.05	0.06
Euryarchaeota (0.6 %)	*Methanobacteria (0.7 %)*	*Methanobacteriales (0.7 %)*	*Methanobacteriaceae*	*Methanosphaera*	0.45	0.18
				Methanobrevibacter	0.50	0.16
Fibrobacteres (0.2 %)	*Fibrobacteria (0.2 %)*	*Fibrobacterales (0.2 %)*	*Fibrobacteraceae*	*Fibrobacter*	0.15	0.28
Firmicutes (67.7 %)	*Bacilli (8.4 %)*	*Lactobacillales (7.1 %)*	*Streptococcaceae*	*Streptococcus*	1.63	5.58
			Lactobacillaceae	*Lactobacillus*	4.63	2.58
		Turicibacterales (1.3 %)	*Turicibacteraceae*	*Turicibacter*	1.12	1.52
	Clostridia (58.8 %)	*Clostridiales (58.8 %)*	*Lachnospiraceae*	*Lachnospira*	0.95	0.08
				Coprococcus	0.60	0.58
				Other	0.05	0.14
				Epulopiscium	0.00	0.00
				Dorea	0.17	0.30
				Blautia	0.35	0.28
				Shuttleworthia	3.57	0.52
				Roseburia	0.30	0.20
				Butyrivibrio	0.03	0.00
			Ruminococcaceae	*Oscillospira*	0.88	1.78
				Faecalibacterium	0.03	0.00
				Ruminococcus	2.57	2.70
				-	7.90	12.62
			Clostridiaceae	*SMB53*	4.92	1.74
				Clostridium	3.55	2.20
				-	22.87	12.90
			Veillonellaceae	*Anaerovibrio*	0.03	0.02
				Phascolarctobacterium	0.07	0.04
				Succiniclasticum	0.00	0.02
				Megasphaera	0.73	0.08

			Christensenellaceae	-	0.15	1.82
			Mogibacteriaceae	-	0.15	0.20
			Peptostreptococcaceae	-	0.80	0.28
			Peptococcaceae	*Peptococcus*	0.00	0.04
	Erysipelotrichi (0.5 %)	*Erysipelotrichales (0.5 %)*	*Erysipelotrichaceae*	*Catenibacterium*	0.03	0.02
				[Eubacterium]	0.03	0.02
				L7A_E11	0.13	0.06
				Bulleidia	0.07	0.08
				p-75-a5	0.23	0.18
Planctomycetes (0.9 %)	*Planctomycetia (0.9 %)*	*Pirellulales (0.9 %)*	*Pirellulaceae*	-	0.55	1.28
Proteobacteria (0.6 %)	*Betaproteobacteria (0.2 %)*	*Tremblayales (0.2 %)*	-	-	0.38	0.02
	Deltaproteobacteria (0.0 %)	*Desulfovibrionales (0.0 %)*	*Desulfovibrionaceae*	*Desulfovibrio*	0.03	0.00
	Epsilonproteobacteria (0.1 %)	*Campylobacterales (0.1 %)*	*Campylobacteraceae*	*Campylobacter*	0.03	0.06
	Gammaproteobacteria (0.3 %)	*Aeromonadales (0.1 %)*	*Succinivibrionaceae*	*Succinivibrio*	0.08	0.06
		Enterobacteriales (0.2 %)	*Enterobacteriaceae*	-	0.30	0.00
Spirochaetes (13.5 %)	*Spirochaetes (13.5 %)*	*Spirochaetales (13.5 %)*	*Spirochaetaceae*	*Treponema*	12.48	14.66
Synergistetes (0.0 %)	*Synergistia (0.0 %)*	*Synergistales (0.0 %)*	*Dethiosulfovibrionaceae*	-	0.00	0.08
Tenericutes (0.1 %)	*Mollicutes(0.1 %)*	*RF39 (0.1 %)*	-	-	0.05	0.12
TM7 (0.1 %)	*TM7-3 (0.1 %)*	*CW040 (0.1 %)*	*F16*	-	0.15	0.06
Other (0.6 %)	*Other (0.6 %)*	*Other (0.6 %)*	*Other*	*Other*	0.55	0.62
Verrucomicrobia (0.6 %)	*Opitutae (0.0 %)*	*Cerasicoccales (0.0 %)*	*Cerasicoccaceae*	-	0.00	0.04
	Verruco-5 (0.4 %)	*WCHB1-41 (0.4 %)*	*RFP12*	-	0.35	0.42
	Verrucomicrobiae (0.2 %)	*Verrucomicrobiales (0.2 %)*	*Verrucomicrobiaceae*	*Akkermansia*	0.00	0.46
WPS-2 (0.1 %)	*- (0.1 %)*	*- (0.1 %)*	-	-	0.08	0.10

5.4.2 Proportions of OTU's at Phylum, Class, Order and genus levels

Dominant phyla detected were *Firmicutes* (67.7 %), *Bacteroidetes* (14.8 %) and *Spirochaetes* (13.5 %) as shown in Table 5.1. *Fibrobacteres*, *TM7*, *Verrucomicrobia*, *Cyanobacteria*, *Synergistetes*, *WPS-2*, *Tenericutes*, *Planctomycetes*, *Proteobacteria*, *Actinobacteria* and *Euryarchaeota* were below 1 % and accounted for 4.1 % of total reads. The *Euryachaeota,* a member of the *Archea* kingdom, comprised 0.7 % of the phyla of which LW x LR had 0.5 % and the SAWIP had 0.2 %. In the *Firmicutes* phylum*, Clostridia* was the most dominant class (58.8 %), followed by *Bacilli* (8.4 %) while the *Bacteroidetes* phylum comprised only the *Bacteroidia* class (14.8 %). The *Spirochaetes* phylum was represented by the *Spirochaetes* class, *Spirochaetales* order, *Spirochaetaceae* family and *Treponema* genus comprising 13.5 % of all OTU's in each of the different taxonomic groups. The *Bacilli* class split into *Turicibacterales* (1.3 %) and *Lactobacillales* (7.1 %). Four families *Clostridiaceae* (24.0 %), *Ruminococcaceae* (14.3 %), *Spirochaetaceae* (13.6 %) and *Lachnospiraceae* (8.0 %) were the most abundant. The other families above 5 % included the *Bacteroidales* S24-7 (7.0 %).

5.4.3 Breed and diet effects on OTU's with cellulolytic and hemicellulolytic activities

About 11.3 % OTU's from SAWIP and 12.7 % OTU's from LW x LR on the HMC diet, 11.6 % OTU's from SAWIP and 7.9 % OTU's from LW x LR on the CON diet were not assigned to any family. At the genus level, 56.3 % OTU's from SAWIP on HMC, 50.0 % OTU's from LW x LR on HMC, 53.5 % OTU's from SAWIP on CON and 58.8 % OTU's from LW x LR on CON were not assigned to any genus. The breed responses of the OTU's at the phylum level to the two diets are in Table 5.2 and Figure 5.3. Although there were differences in the abundance of *Firmicutes*, *Bacteroidetes*, and *Spirochaetes* between breeds and between diets, these were not significant (Table 5.2). The ratio of *Bacteroidia* to *Clostridia* in SAWIP on the HMC diet (0.37) was similar to those on the CON diet (0.39). In LW x LR on the HMC diet the ratio of the *Bacteroidia* to *Clostridia* was higher than those on the CON diet (0.24 vs 0.1). Table 5.3 and Figure 5.4 show the differences in the abundance of the genera between the breeds and the diets. There was a breed x diet interaction ($P < 0.05$) for *Oscillospira*. *Oscillospira* increased in LW x LR as diet changed from CON to HMC while it decreased in the SAWIP. *Verrucomicrobia Opitutae* was detected only in SAWIP on the HMC diet. There were no differences in breed, diet and breed x diet interaction in abundance of the other families.

Table 5.2 Relative abundances (%) of dominant faecal bacterial phyla in Large White × Landrace crossbred pigs (LW x LR) and South African Windsnyer-type Indigenous pigs (SAWIP) pigs fed control (CON) and high maize cob level (HMC) diets

	HMC diet		CON Diet	
Phylum	SAWIP	LW x LR	SAWIP	LW x LR
Bacteroidetes	19.8	14.8	19.2	6.9
Firmicutes	62.0	70.2	61.2	75.1
Spirochaetes	14.4	11.1	15.1	13.9

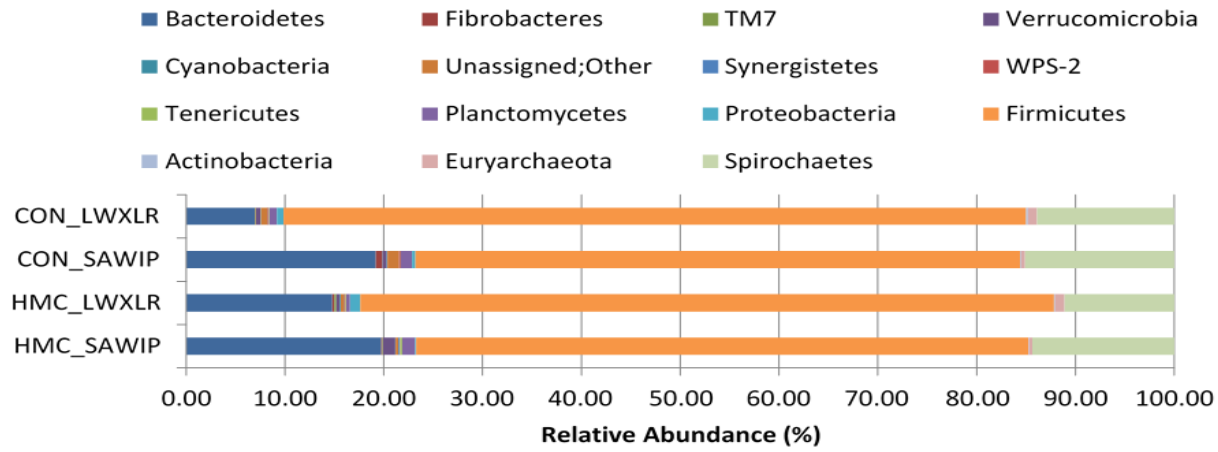

Figure 5.3 Relative abundance (%) of OTUs observed at the phylum level in the LW x LR and SAWIP pigs in the distal gut microbiota fed control (CON) and high maize cob level (HMC) diets

Table 5.3 Relative abundances (%) of dominant faecal bacterial genera in Large White × Landrace crossbred pigs (LW x LR) and South African Windsnyer-type Indigenous pigs (SAWIP) pigs fed control (CON) and high maize cob level (HMC) diets

	HMC		CON		P values		
Genus	SAWIP	LW x LR	SAWIP	LW x LR	Brd	Diet	BrdxDiet
Oscillospira	0.9	1.2	3.1	0.5	0.654	0.885	0.02
Clostridiaceae genus	14.1	15.3	11.1	30.5	0.654	0.885	0.534
Christensenellaceae genus	1.9	0.1	1.7	0.2	0.129	0.953	0.534
Prevotella	1.6	4.1	2.9	0.7	0.904	0.885	0.534
SMB53	1.9	3.9	1.4	6.0	0.654	0.885	0.794
Bacteroidales genus	1.6	1.7	1.4	0.4	0.686	0.885	0.794
Ruminococcaceae genus	13.6	8.1	11.2	7.7	0.654	0.885	0.794
Akkermansia	0.8	0.0	0.0	0.0	0.654	0.885	0.794
Parabacteroides	6.5	0.2	0.1	0.03	0.654	0.885	0.794
Streptococcus	6.3	2.5	4.5	0.7	0.654	0.885	0.794
Lactobacillus	0.5	5.3	5.7	4.0	0.713	0.889	0.794
YRC22	1.2	1.9	2.0	0.3	0.819	0.889	0.794
Pirellulaceae genus	1.3	0.4	1.1	0.8	0.654	0.961	0.794
Clostridium	2.8	3.4	1.3	3.6	0.654	0.953	0.823
S24-7 genus	7.7	5.6	10.2	4.6	0.654	0.969	0.865
Turicibacter	1.8	0.7	1.1	1.5	0.800	0.961	0.896
Ruminococcus	2.6	3.1	2.9	2.0	0.904	0.944	0.946
Clostridiales genus	9.0	9.1	8.6	6.2	0.819	0.885	0.946
Lachnospiraceae genus	4.1	4.1	3.2	3.9	0.904	0.953	0.984
Treponema	14.4	11.1	15.1	13.9	0.881	0.961	0.984

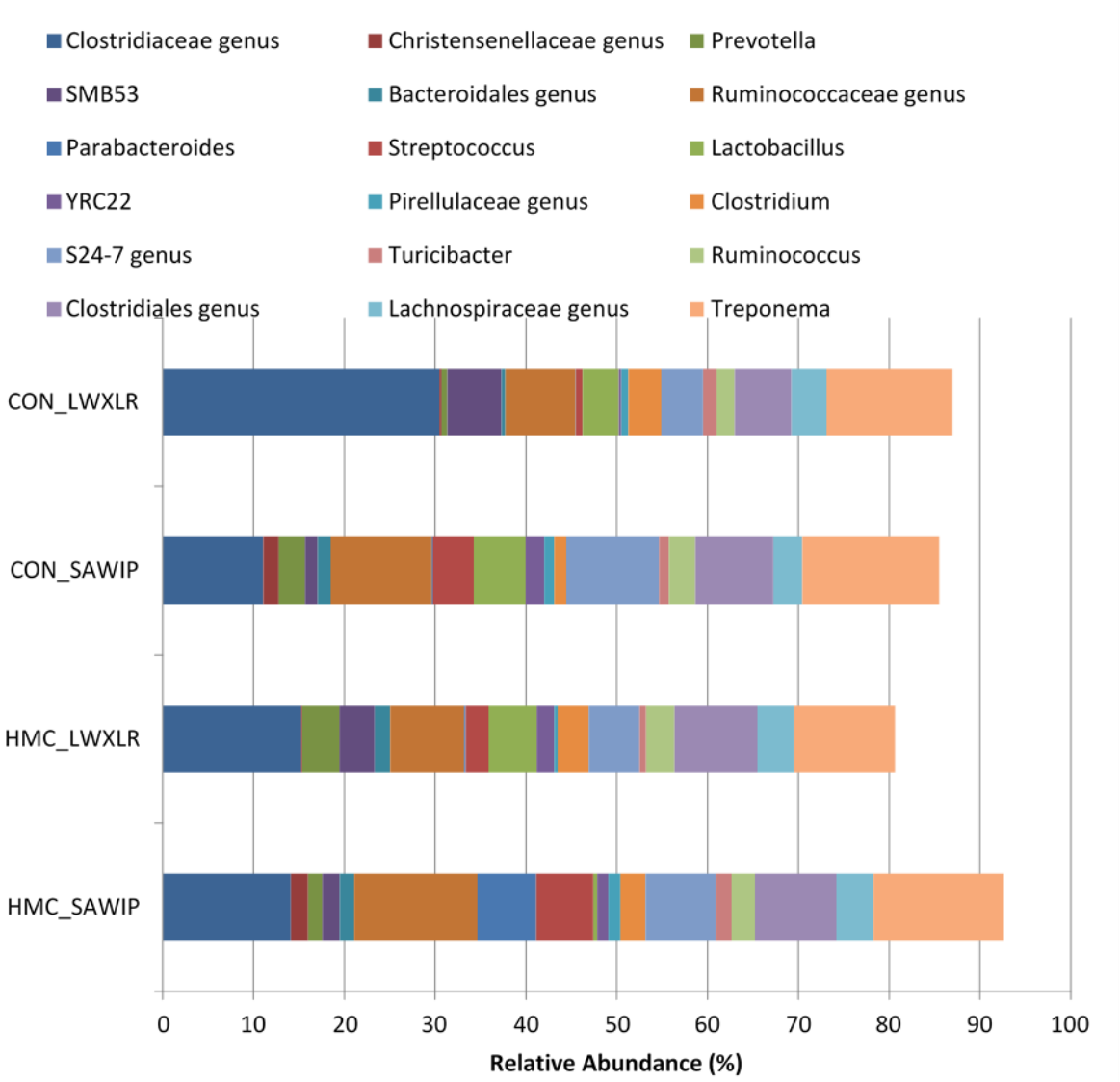

Figure 5.4 Relative abundance (%) of OTUs observed at the genus level in the LW x LR and SAWIP pigs in the distal gut microbiota fed control (CON) and high maize cob level (HMC) diets

5.5 Discussion

The core microbial communities as well as the impact of a diet containing ensiled maize cobs on microbial profiles in two pig breeds, the LW x LR and SAWIP, were compared. Leser *et al*. (2002) previously studied the phylogenetic diversity of the intestinal bacterial community in Danish commercial pigs using 16S rDNA sequence analysis. There are however no similar studies to date on the microbiomes of indigenous pigs from Southern Africa. The coverage of available phylotypes was extensive as reflected in the rarefaction curves. The rarefaction curves began with steep slopes, which flattenned to a plateau as fewer new species were

discovered per sample. Although rarefaction estimates showed differences in number of OTUs identified between breeds, treatments, and their interactions, there were no significant differences in microbial diversity. These observations contradict Hill *et al*. (2005) who posited that each individual pig harbors its own specific and unique bacterial composition, even if the animals receive the same diet, stay in the same environment, and are siblings.

Pigs fed on high-fibre diets require 3 to 5 weeks to adapt to the digestibility of resistant non-starch polysaccharide monomers (Longland *et al*., 1993). In this study, the pigs were on the same diet for at least 8 weeks, so the intestinal microbes were well adapted and in a stable flux. It had been expected that the ensiling of the maize cobs in the current study would have improved their fermentability. The outliers observed in both the LW x LR and SAWIP breeds, may explain the lack of statistically significant differences between breeds or diets. In previous studies, bacteria with cellulolytic and hemicellulolytic activities were reported to increase on diets containing high levels of dietary fibre (Varel & Yen, 1997; Metzler & Mosenthin, 2008) in contrast to the findings in this study. Upadrasta *et al*. (2013) reported significant changes in the proportions of bacterial communities observed at all OTU levels between the cider yeast supplemented group and control diet group at day 21 in weaner pigs. Varel *et al*. (1985) however reported that there was no increase in cellulolytic bacteria when a 20 % maize cob diet was fed to sows.

The notion of genotypic influences concerning variations in intestinal community phylogenetic diversity in pigs was proposed (Varel *et al*., 1988). The 835 OTUs identified in the samples from LW x LR and SAWIP were similar. Many of the OTU's also matched those identified in pigs in other studies (Varel & Yen, 1997; Lester *et al*., 2002; Upadrasta *et al*., 2013). It is important to recognize the shortcomings of PCR amplification of 16S rDNA followed by pyrosequencing when evaluating this data. Biases based on variation in DNA extraction, primer specificity, PCR amplification are inherent making comparisons to other studies difficult (Freitas *et al*., 2012). Meanwhile a significant proportion of OTU's were not assigned to any family or genus and these will need to be investigated further in future studies.

The SAWIP and LW x LR breeds are obese and lean pigs respectively and differences in their energy metabolism may reflect as differences in microbiome composition. The observed different proportions of *Euryacheota,* a group of methanogenic archea in the LW x LR and SAWIP might be one explanation for their tendencies towards leaness and obesity, respectively. A relationship between the composition and abundance of intestinal tract methanogenic communities, and the host's energy metabolism, and the fatness or leanness of the host is accepted (Su *et al*., 2014). Luo *et al*. (2012) reported that Landrace pigs exhibited significantly more methanogen diversity than Erhualian pigs. Erhualian pigs are Chinese indigenous pigs that have not been selected for lean growth and tend to be obese (Luo *et al*., 2012); making them somewhat similar to the SAWIP. Meishan pigs, an indigenous Chinese obese breed, showed an increased relative abundance of *Firmicutes* and lower numbers of *Bacteroidetes* than Landrace pigs (Guo *et al*., 2008). Although in this study the abundance of *Firmicutes*, *Bacteroidetes*, and *Spirochaetes* between breeds and diets were not significantly different, the ratios of *Bacteroida* to *Clostridia* in each breed are suggestive of genotype influences.

The *Oscillospira* genus of the order *Clostridiales* and phylum *Firmicutes* increased in LW x LR pigs as diet changed from CON to HMC but decreased in the SAWIP. The functional capabilities of the genus *Oscillospira* have not been determined, but it is likely that it plays a role in fibre fermentation due to its presence in numerous rumen systems and its greater abundance in hosts that are fed fresh forage (Mackie *et al*., 2003). The presence of *Verrucomicrobiae* in SAWIP on HMC diet and not in other treatments is currently difficult to explain. This is because the phylum *Verrucomicrobia* has a widespread distribution, and is known to be one of the most common and diverse phyla in soil, aquatic habitats and in the gut of eukaryotes (Lee *et al*., 2009; Kielak *et al*., 2010). However, since members of this phylum have been difficult to cultivate, it has hampered studies on understanding its role (Kielak *et al*., 2010). Some members of the *Verrucomicrobia* phylum have been reported to oxidize methane and use methane as a sole source of carbon and energy, making them the only known aerobic methanotrophs outside the *Proteobacteria*, and the only extreme acidophilic methanotrophs known (Dunfield *et al*., 2007; Islam *et al*., 2008). A related bacterium, strain *VeGlc2* of the order *Verrucomicrobiales,* was shown to ferment glucose to acetate, propionate, succinate, and CO_2 through the Embden-Meyerhof-Parnas pathway (Janssen, 1998).

5.6 Conclusions

Analysis of faecal microbiomes revealed differences occurring between the LW x LR and SAWIP breeds but not between the CON and HMC diets. Some of these differences might explain the enhanced ability of the SAWIP to digest fibrous diets better than the LW x LR breed. Since the gut microbiome impacts on nutrient utilisation there is need to define the relationship between the faecal microbiome and pigs' efficiency in digesting fibrous diets. This may be reflected as differences in growth performance and carcass measurements.

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Chapter 6

6 *Serum metabolite profiles, liver histometry and proteomic analysis of South African Windsnyer-type indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) crosses fed diets containing ensiled maize cobs*

Abstract

A study to compare serum metabolite and liver histometry responses in indigenous pigs and commercial pigs fed diets containing ensiled maize cobs was undertaken. In addition, proof of principle on the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis matrix-assisted laser desorption ionization mass spectrometry (SDS-PAGE /MALDI MS) workflow to determine differences in serum and liver protein profiles of pigs fed high fibre diets was established. Twenty-four Large White × Landrace crossbred pigs (LW x LR) and 15 South African Windsnyer-type Indigenous pigs (SAWIP) were assessed in the study. They were fed a control diet (CON), a low ensiled maize cob inclusion (LMC) and a high ensiled maize cob inclusion (HMC) diet in a completely randomized block design. Blood urea nitrogen concentration was greater ($P < 0.05$) in the SAWIP than LW x LR. Creatinine, phosphorus, alkaline phosphatase (ALP), cholesterol and serum α-amylase (AMYL) values were greater ($P < 0.05$) in LW x LR than in the SAWIP. There were breed x diet interactions ($P < 0.05$) for alanine aminotransferase (ALT) and AMYL. The LW x LR had greater values of ALP and AMYL than the SAWIP. Intensity of 22 kDa protein bands in LW x LR on the HMC diets was greater ($P < 0.05$) than in SAWIP on HMC diet. Protein bands of molecular weight (MW) 22 kDa were present in SAWIP on CON and HMC diets and absent from LW x LR on HMC diets. A 24 kDa MW protein band was observed more consistently in the LW x LR on the CON diets and in SAWIP on the HMC diet than in the LW x LR on the HMC diet. Protein bands of MW 36 kDa were present in SAWIP on HMC diets and absent from LW x LR on HMC diets. Two proteins, *guanidinoacetate N-methyltransferase*-like isoform 1 associated with creatine biosynthetic and *catalase*, which is involved in cholesterol metabolic processes were identified. There were differences in serum and liver proteins and in serum metabolite levels that were diet and breed related. This suggests that proteomics could play a role in evaluating the performance of pigs under different feeding regimes. A proof of principle to assess serum and liver protein profiles of pigs fed a a high fibre diet using a sodium dodecyl sulphate polyacrylamide gel electrophoresis matrix-assisted laser desorption ionization mass spectrometry (SDS-PAGE /MALDI MS) workflow was established.

Key words: biomarkers, fibre, mass spectrometry, pig genotype

6.1 Introduction

There is a need to assess how differences in feed intake and digestibility of high fibre diets present at the level of cellular biology. This could be done through proteomics which can assess thousands of proteins produced by tissues at a specific time and under given conditions (Vaidyanathan. 2005). Wang *et al.* (2009) showed that weanling pigs supplemented with zinc oxide up-regulated 22 and down-regulated 19 protein spots in the jejunum related to energy metabolism. Zhong *et al.* (2011) also demonstrated that conjugated linoleic acid (CLA) in pig diets positively influenced the expression of proteins related to energy and amino acid metabolism and fatty acid oxidation and synthesis. Proteomics could explain the mechanisms behind the physiological and morphological changes in pigs fed on fibrous diets and identify biomarkers for selection purposes. For example, such biomarkers could be used to identify breeds and individual pigs that utilize cheaper fibrous feed ingredients leading to reduced pork production costs. In addition, understanding of molecular and cellular mechanisms would enable the design of rational and innovative approaches to formulations of feed and use of feed additives to ensure the health and well-being of the pigs (Shirazi-Beechey *et al.,* 2011).

High fibre diets are likely to have a direct impact on serum and liver metabolites and proteins but since these are not homogenous tissues, there are no easy, robust, and sensitive protocols for identification of dietary-induced changes in proteins. Traditionally, serum metabolite assays have been the main tool to assess physiological responses to diets and diseases in animals (Kaneko, 1989; Varghese *et al*., 2012). These assays have generally encompassed metabolic markers, (glucose, cholesterol, triglycerides), markers of liver health and function (total protein, albumin, alkaline phosphatase, alanine aminotransaminase, total bilirubin, globulins) and other serum markers (α-amylase, blood urea nitrogen, calcium, creatinine, phosphorus). However, serum is also a rich source for molecular protein profile characterization. Serum is however difficult to work with at the molecular level because of high globulin and albumin levels which interefere with detection of proteins of lower concentrations. Various techniques have been used to reduce the globulins, including the use of affinity chromatography columns that bind the top 6 to 12 abundant globulins (Chen *et al.,* 2005). The liver is central to metabolic activities in the body and it has also been assessed through morphological changes. However, the proteome of the pig liver remains largely unexplored (Yi *et al.,* 2013). In this study, it was hypothesized that a diet containing high levels of ensiled maize cobs modifies the liver morphology and serum metabolites and proteome differently in South African Windsnyer-type indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) crosses. The objective of the study was to compare serum metabolite and proteomic profiles and liver histometry changes in SAWIP and LW x LR crosses fed diets containing ensiled maize cobs to those without.

6.2 Materials and Methods

6.2.1 Pigs, housing and experimental design

The experimental procedures described in this study were approved by the Animal Ethics Committee of the Agricultural Research Council, Animal Production Institute (ARC-API). The pigs, housing conditions and diets are described in Chapter 5, Sections 5.2.1 and 5.2.2. Measurements of weight gain, feed intake and carcass measurements were taken in eight LW x LR and five SAWIP pigs in the three diets (CON, LMC and HMC) diet as part of a growth performance study. In order to maximize use of resources, histometry and serum proteomic analysis were only evaluated in three pigs of each breed on the CON and HMC diets as these two diets were likely to evoke the most divergent morphological and proteomic responses. In the same vein, liver proteomic analyses were only done in the diet where breed differences were expected to be most marked (the HMC diet) to compare the effect of two different protein concentrations on protein separation and also identify specific proteins that were differentially expressed. Each pig was allocated feed daily in the morning based on their ability to finish and 10 % adjustments were made on those that managed to finish their daily allocation. Refusals were weighed in every morning and subtracted from feed offered to determine intake. The feeders were checked and adjusted twice each day to ensure constant access to fresh feed and minimize any possible wastage. Water was freely available through nipple drinkers.

6.2.2 Sampling procedures

At the end of the growth study, the pigs were taken to the abattoir situated less than one kilometre from the pens at around 0800 hours. The pigs were processed according to the routine abattoir procedures after an ante-mortem inspection and rest for the pigs before slaughter. Prior to slaughter, the pigs were electrically stunned with an electrical stunner set at 220 V and 1.8 A with a current flow for 6 s and exsanguinated within 10 s of stunning. During exsanguination, 10 ml blood samples were collected in plain tubes and left to clot for about 1 h after which they were centrifuged for 10 min at 2 000 x g at room temperature to separate the serum as supernatant. An aliquot of the serum was then kept at -20 °C for biochemical analysis while another was then kept at -80 °C for proteomic analysis. Dehairing and evisceration of carcasses followed and the gastrointestinal tracts were set aside for sample collection. On receiving the clean offals, the fifth lobe of liver was identified, and two portions of approximately 5 g were excised, one was put in a plastic bottle containing 10 % neutral buffered formaldehyde for Haematoxylin and Eosin (H & E) staining and another was put into a small plastic tub and immediately snap-frozen in liquid nitrogen for proteomic analyses. The proteomic analyses samples were kept at -80 °C freezer until analysis. Care was taken not to cross-contaminate samples by cleaning dissection instruments with 70 % ethanol and changing gloves between dissections.

6.2.3 Laboratory procedures

6.2.3.1 Serum analyses

Determination of serum metabolite and enzyme levels was done using IDEXX Vettest® Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, ME. USA). The IDEXX Vettest® Chemistry Analyzer employs dry-slide technology that uses a potentiometric end-point. The analyte in the sample catalyzes a reaction sequence to yield products that absorb light at wavelengths in various regions (340-680 nm), diffuses into an underlying layer, and are monitored by reflectance spectrophotometry. The dry slide technology minimizes interferences from lipemic, icteric and hemolyzed samples. The General Health Profile (GHP) evaluated comprised albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), serum α-amylase (AMYL), blood urea nitrogen (BUN), calcium (CAL), cholesterol (CHOL), creatinine (CREAT), globulins (GLOB), glucose (GLU), phosphorus (PHOS), total bilirubin (TBIL) and total protein (TP).

6.2.3.2 Histometry of the liver

Hepatic tissues from pigs on the CON and HMC diets were fixed in 10 % neutral buffered formaldehyde and embedded in paraffin. Paraffin sections were stained with H & E stain. The liver sections were then assessed using a Zeiss AxioSkop 2 light microscope. Images were captured and analysed using AxioVision 4 Imaging Solutions software (AxioVs40 V 4.8.1.0, Carl Zeiss, Jena, Germany). Images were initially encoded on 24-bits per pixel on three channels (red, green, and blue). For the four experimental groups (SAWIP CON, SAWIP HMC, LW x LR CON and LW x LR HMC), a quantitative assessment of the hepatocyte area, gray matter, and number of hepatocyte per unit area was carried out on the images using ImageJ (Wyne Rasband, National Institutes of Health, Bethesda, USA).

6.2.3.3 Protein extraction from pig serum

6.2.3.3.1 Pig serum albumin depletion using trichloroacetic acid (TCA)/acetone method

Eleven serum samples (3 from LW x LR CON diet, 3 from LW x LR HMC diet, 2 from SAWIP CON and 3 from SAWIP HMC) were retrieved from -80 °C storage and immediately thawed on ice. From each of the thawed and vortexed samples 50 µl was transferred to a new tube. A protocol adapted from Chen *et al.* (2005) was used to precipitate the samples. Briefly, 50 µl of distilled deionized water (dH_2O) was added to 50 µl of serum and mixed by vortexing. To this mixture 400 µl of ice-cold 10 % TCA/acetone (v/v) were added and mixed by vortexing. The tubes were incubated at -20 °C for 90 min. After 90 min the samples were removed from -20 °C, centrifuged at 16 000 x g at 4 °C, for 15 min. The supernatant was carefully removed and collected into new tubes. Ice cold acetone (0.5 ml) was added to the pellet to wash it. The tube with the pellet was incubated on ice for 15 min. The tubes were then centrifuged at 16 000 x g at 4 °C, for 15 min.

The supernatant was removed and added in the supernatant collection tube. The pellet was air-dried briefly for 15-30 s, excess acetone was blotted with a paper towel and the tube was capped immediately. The pellet was resuspended in 100 µl lysis buffer (9 M urea, 2 M thiourea and 4 % CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate)). To the supernatant collection tube 0.5 ml of ice-cold acetone was added to precipitate and remove TCA. The tubes were incubated at -20 °C for 90 min. After incubation the tubes were centrifuged at 16 000 x g at 4 °C for 15 min. The supernatant was carefully removed and discarded. The pellet was air-dried briefly for 15-30 s, excess acetone was blotted using paper towel and the tube was capped immediately. The pellet was then resuspended in 100 µl lysis buffer and as aliquots at -20 °C. Protein concentration was determined using the Bradford method with bovine serum albumin (BSA) as a standard (Bradford, 1976) as described in Section 6.2.3.5.

6.2.3.4 *Protein extraction from pig liver*

Three biological replicates from frozen liver samples for both the LW x LR and SAWIP breeds on the HMC diets were included in protein extraction. A biological replicate represented a pool of three samples from different pigs in each breed on the same diet. The samples were retrieved from the -80 °C storage and about 0.2 g cut immediately from each liver and put in a small mortar. They were then ground to a fine powder using a pestle and mortar under liquid nitrogen flow and mixed thoroughly before being divided into two 0.35 g portions per breed and put into different micro-tubes. The samples were then suspended in 0.5 ml lysis buffer and centrifuged at 13, 200 x g for 30 min at room temperature. After this, 200 µl of supernatant was collected to fresh tubes and mixed with 800 µl absolute acetone and incubated at -21 °C for an hour. This mixture was centrifuged at 13,200 x g for 10 min and the supernatant was decanted and the pellet air dried. The pellet was then washed with 800 µl of 80 % acetone, vortexed, and centrifuged at 13,200 x g for 5 min, the supernatant decanted and the pellet air dried, repeated three times. The pellet was then re-suspended in 100 µl lysis buffer. Protein concentration was determined using the Bradford method with BSA as a standard (Bradford, 1976) as described in Section 6.2.3.5.

6.2.3.5 Protein quantification

Protein concentration was determined using the Bradford method with BSA as a calibration standard (Bradford, 1976). Briefly, 0, 1, 2, 4, 8 and 10 µl of a stock solution of BSA 5 mg/ml were mixed with 10 µl of 0.1 M HCl, 80 µl of dH_20, 900 µl of 20 % (v/v) Bio-Rad Dye reagent and extraction buffer (9 M urea, 2 M thiourea, 40mM Tris (pH 8.5) and 4 % CHAPS) to a final volume of 1 ml to obtain increasing concentrations of BSA ranging from 0 to 50 µg. The absorbance of the mixture was measured at 595 nm and a calibration curve generated, using a Genesis 5 Spectrophotometer (Milton Roy, Groton, CT, USA). A total of 5 µl of liver and serum protein extracts were mixed with 10 µl of 0.1 M HCl, 80 µl of dH_20, 900 µl of 20 % (v/v) Bio-Rad Dye reagent and 5 µl extraction buffer to a final volume of 1 ml. The absorbance of the mixture was

measured at 595 nm. The concentrations of the protein extracts were then obtained by extrapolating from the BSA standard curve.

6.2.3.6 One dimensional polyacrylamide gel electrophoresis (1D-PAGE)

6.2.3.6.1 Serum samples

The eleven serum protein extracts (3 from LW x LR CON diet, 3 from LW x LR HMC diet, 2 from SAWIP CON and 3 from SAWIP HMC) were separated according to their molecular weight by 1D-PAGE in two gel combination runs. The first gel compared protein extracts from the LW x LR CON, LW x LR HMC and the SAWIP CON diets while the second gel compared extracts from the LW x LR HMC, SAWIP CON and SAWIP HMC diets. Serum protein extracts were mixed with an equal volume of sodium dodecyl sulphate (SDS) loading dye (50 mM Tris (pH 6.8), 10 mM Dithiothreitol (DTT), 5 % SDS (w/v), 0.1 % (w/v) bromophenol blue, 20 % (v/v) glycerol) (5 µl:5 µl) by vortexing and heating for 5 min at 95 °C. Samples were then centrifuged at 16, 000 x g for 2 min at room temperature, before loading 20 µg on to wells in a Mini-Protean III® Cell gel casting system (Bio-Rad Laboratories, Hercules, CA, USA). A 12 % resolving gel (6.45 ml, dH_2O; 4.5 ml, 40 % acrylamide; 3.8 ml, 1.5 M Tris pH 8.8; 0.15 ml, 10 % SDS; 0.15 ml, 10 % Ammonium persulphate (APS) and 0.006 ml N,N,N',N'-tretramethylethylenediamine (TEMED)) and a 4 % stacking gel (3.6 ml, dH_2O; 0.625 ml, 40 % acrylamide; 0.63 ml, 1.5 M Tris pH 6.8; 0.05 ml, 10 % SDS; 0.05 ml, 10 % APS and 0.005 ml TEMED) were used for the separation, The resolving gel was allowed to polymerize for 20 min and the stacking gel for 10 min at room temperature. A running buffer (576 g glycine, 121.1 g Tris base, 40 g SDS topped up to 4 L with dH_20) was prepared and used to fill the Mini-Protean III® Cell gel tank (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was conducted at 100 V past the stacking gel after which the voltage was increased to 140 V from a PowerPac Basic Power Supply™ (Bio-Rad Laboratories, Hercules, CA, USA) at room temperature until the dye reached the bottom of the gel. The gels were stained with Coomassie brilliant blue R-250 stain for visualisation and digitalized images of the gels were captured using Pharox FX® imager as described in Section 6.2.3.8.

6.2.3.6.2 Liver samples

Equal volumes of liver samples and loading buffer (15 µl) were vortexed and heated for 5 min to 95 °C. Samples were then centrifuged at 16, 000 x g for 2 min at room temperature, before loading two different concentrations (20 and 30 µg) of protein per well alternately in a 10 well Mini-Protean III® Cell gel casting system (Bio-Rad Laboratories, Hercules, CA, USA) for protein profiling using the same protocol as described for serum protein extracts in 6.3.3.3.5.1. Profiles of two liver protein extract concentrations (20 vs 30 µg) from three biological replicates for both the LW x LR and SAWIP breeds on the HMC diets were compared. The

gels were stained with Coomassie brilliant blue R-250 stain for visualisation and digitalized Images of the gels were captured using Pharox FX® imager as described in Section 6.2.3.8.

6.2.3.7 Two dimensional gel electrophoresis (2D-PAGE)

6.2.3.7.1 Sample preparation

Aliquots containing 6-9 µg/µl total liver protein extract representing the two breeds on the HMC diet stored at -20 °C were thawed on ice and diluted in rehydration buffer (9 M Urea; 2 M thiourea; 4 % CHAPS; 0.2 % Ampholyte; 50 mM DTT; a pinch of bromophenol blue) to a total volume of 125 µl constituting 250 µg of each sample. The sample was then vortexed and centrifuged at 16, 000 x g for 2 min at room temperature. .

6.2.3.7.2 Rehydration of immobilized pH gradient strips

Dry polyacrylamide immobilized pH gradient (IPG) strips (Amersham Pharmacia Biotech) measuring 70 mm in length with a pH gradient of 3-10 were used. The rehydration buffer (125 µl) containing protein constituting 250 µg of each sample were loaded into the rehydration tray and after careful removal of the protecting cover foil, the IPG strips were gently positioned on top of it, avoiding trapping air bubbles. Each strip was covered with mineral oil to prevent dehydration and allowed to passively rehydrate overnight at room temperature.

6.2.3.7.3 Isoelectric focusing (IEF)

After rehydration, the IPG strips were removed from the rehydration tray, rinsed with dH_2O and excess water drained on filter paper. Wicks were prepared by wetting with 10 µl dH_2O and placed on each end of the strip to absorb excess salt. The IPG strips were then loaded on an Ettan™IPGphor™ II IEF machine (GE Healthcare, Biosciences AB, Uppsala, Sweden), gel side facing upwards. The IPG strips were overlaid with mineral oil and focused at 30 V for 3 h, 100 V for 1 h, 200 V for 1 h, 500 V for 1 h, 1,000 V for 1 h and finally at 8,000 V for 11 h to obtain approximately 90,000 Vh at room temperature to separate the proteins according to their p*I*, the pH at which a protein carries no net charge and will not migrate any further in an electrical field. At the end of the run, the strips were placed in an equilibration tray for equilibration prior to the second dimension resolution by SDS-PAGE.

6.2.3.7.4 Equilibration

Once IEF was completed, the strips were equilibrated in SDS equilibration buffer I (6 M urea; 0.375 ml Tris HCl pH 8.8; 2 % SDS; 20 % glycerol; 2 % (w/v) DTT; with dH_2O making it up to 50 ml) by inserting them with

the gel side up in the rehydration tray, put on a shaking plate for 15 min and rinsed. The process was repeated with SDS equilibration buffer II (6 M urea; 0.375 Tris HCl pH 8.8; 2 % SDS; 20 % glycerol; 2.5 % (w/v) iodoacetamide; with dH_2O making it up to 50 ml).

6.2.3.7.5 Second dimension of 2D-PAGE by SDS-PAGE

In the second dimension, proteins resolved on IPG strips were applied to a 12 % SDS-PAGE gel and separated by molecular mass. SDS-PAGE was performed using a precast gel (8.6 x 6.7 x 0.1 cm; Bio-Rad Laboratories, Hercules, CA, USA). The IPG strip was applied to the gel and sealed with Agarose sealing solution (0.5 % Agarose (w/v) in 1X SDS-PAGE running buffer with a hint of bromophenol blue). The second dimension electrophoresis was carried out at 100 V past the stacking gel after which the voltage was increased to 140 V using a PowerPac Basic Power Supply™ (Bio-Rad Laboratories, Hercules, CA, USA). The gels were then stained with Coomassie stain for visualisation as described in Section 6.2.3.8.

6.2.3.8 Detection of proteins by gel staining with Coomassie brilliant blue (CBB)

The 1D-PAGE and 2D-PAGE gels were stained with Coomassie brilliant blue (CBB) stain for visualisation. Briefly, the gel was immersed first in CBB staining solution I (10 % (v/v) glacial acetic acid, 2 % (w/v) CBB stock solution, 25 % (v/v) propoan-2-ol) incubated in a microwave for 1 min and left to stain for 1 h with gentle shaking at room temperature. After this, the gel was then incubated in CBB staining solution II (10 % (v/v) glacial aceticacid, 0.25 % (v/v) CBB stock solution, 10 % (v/v) pron-2-ol) and finally in CBB staining solution III (10 % (v/v) glacial acetic acid, 0.25 % (v/v) CBB stock solution, 10 % (v/v) pron-2-ol) each with microwave heating for 1 min and gentle shaking for 1 h at room temperature. The gels were then destained in CBB destaining solution (5 % (v/v) methanol, 10 % (v/v) glacial acetic acid, 1 % (v/v) glycerol) overnight with Kimwipes rolled up into balls added to the trays to speed up the destaining, until an appropriate visualization of protein against background was achieved, thus leaving only the stain linked to the protein spots. Images were then acquired using the PharosFX® Plus molecular image scanner (Bio-Rad Laboratories, Hercules, CA, USA).

6.2.3.9 Image analysis of gels

After scanning of the 1D-PAGE images, they were imported into the analytical software GelAnalyzer 2010 (version: 2010a freeware; www.gelanalyzer.com) which detected lanes and bands. The software also estimated the intensity of each protein band by densitometric quantification, fitted curves to the standard and used the fitted curve to calculate the molecular weight (kDa) of the individual bands of unknown bands and calculated the mobility rate (Rf) of each band. A more precise quantity calibration and evaluation was achieved through the inbuilt background subtraction tool in the software. Digitalized 2D-PAGE images from SAWIP and LW x LR phenotypes were evalauted out manually to analyse similarities and differences in qualities and number of protein spots. Only spots displaying similar patterns but differentially expressed in the two gels were considered for further analyses.

6.2.3.10 In-gel trypsin digestion

Spots of interest were excised from the 2D-PAGE gel manually and transferred into sterile microcentrifuge tubes. The gel pieces were washed twice with 500 µl of 50 mM ammonium bicarbonate for 5 min each time and a third time for 30 min with occasional vortexing. The gel pieces were then destained twice with 500 µl of 50 % (v/v) of 50 mM ammonium bicarbonate and 50 % (v/v) acetonitrile for 30 min with occasional vortexing. These were then dehydrated with 100 µl of 100 % (v/v) acetonitrile for 5 min, and completely dessicated using the Speed Vac SC100 (ThermoSavant, Waltham, MA, USA). The protein in the gel was resuspended and in-gel digested with 120 ng sequencing grade modified trypsin solution for 6 h at 37 °C. The digested proteins were then stored at 4 °C.

6.2.3.11 Protein Identification using MALDI-TOF Mass spectrometry

Prior to identification of digested proteins, 1 µl of each sample was mixed with the same volume of %-cyna-hydroxy-cinnamic (CHCA) matrix and spotted onto a MALDI target plate for analysis by matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) using a Voyager DE Pro Biospectrometry workstation (ABI) to generate a peptide mass fingerprint (PMF). The MALDI-TOF was operated in the positive ion delayed extraction reflector mode for highest resolution and mass accuracy. Peptides were ionized with a 337 nm laser and spectra were acquired at 20 kV acceleration potential with optimised parameters. Close external calibration was employed using the Sequazyme calibration mixture II (ABI) containing angiotensin I, ACTH (1-17 clip), ACTH (18-39 clip) and bovine insulin. This calibration method typically provided mass accuracy of 100 to 200 ppm across the mass range 900 to 5,000 Da. Peptide spectra of accumulated 1,200 shots each were automatically processed for baseline correction, noise removal, and peak deisotoping. The threshold was manually adjusted to 2-8 % base peak intensity. All searches were performed against the National Center for Biotechnology Information (NCBI) and Mass Spectrometry DataBase (MSDB) peptide mass databases using Mass Spectroscopy and Proteomics (MASCOT) (http://www.matrixscience.com/search_form_select.html). Candidate identifications with molecular weight search (MOWSE) scores higher than 85 were automatically considered as positive assignments. For all other assignments of protein spots, the MOWSE score cut off threshold was set to 64. Additionally, positive protein assignments required greater than 10 % sequence coverage. If more than one protein satisfied mentioned threshold criteria, the entry with the highest MOWSE score was assigned. Identified proteins were subsequently validated against the *Malus* EST database from NCBI using full protein sequences and matched peptide sequences to determine similarities. The search criteria required match of at least four peptides from PMF for successful validation.

6.2.4 Data Analysis

Serum metabolites (ALB, ALP, ALT, AMYL, BUN, CAL, CHOL, CREAT, GLOB, GLU, PHOS, TBIL, TP), hepatocyte area, gray-measure and number of hepatocytes per unit area were compared between the breeds and among the diets using ANOVA in the GLM procedures of the statistical package of SAS (SAS Inst. Inc., Cary, NC). All data were tested for normality and homogeneity and comparisons were made to the 95 % significance level and tendencies were considered at $0.05 < P \leq 0.10$.The BUN, TBIL, TP data were log transformed to achieve normality. The protein-band intensity values were compared using ANOVA in the GLM procedures of the statistical package of SAS (SAS Inst. Inc., Cary, NC). For MS/MS MASCOT peptide mass fingerprint searches, MOWSE scores of 64 or greater were considered significant ($P < 0.05$) for each individual fragment.

6.3 Results

6.3.3 Serum metabolites

Serum metabolite measurements in South African Windsnyer-type Indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) fed diets containing ensiled maize at low (LMC) and high (HMC) inclusion levels are presented in Table 6.1. Diet affected alanine aminotransferase (ALT) and α-amylase (AMYL) levels differently in the two breeds as denoted by breed x diet interaction probability value ($P < 0.05$). As maize cobs levels increased there was a decrease in alanine aminotransferase (ALT) levels in SAWIP ($P < 0.05$) while there were no changes in LW x LR ($P > 0.05$). An increase in maize cob level increased amylase (AMYL) levels in SAWIP ($P < 0.05$) while there was no change in LW x LR ($P > 0.05$). Diet did not affect glucose (GLU), blood urea nitrogen (BUN), creatinine (CREAT), phosphorus (PHOS), calcium (CAL), total protein (TP), albumin (ALB), globulins (GLOB), alkaline phosphatase (ALP), cholesterol (CHOL), and total bilirubin (TBIL) differently in the two breeds as shown by breed x diet ($P > 0.05$). Blood urea nitrogen was higher ($P < 0.05$) in SAWIP than LW x LR. The LW x LR had higher values of CREAT, PHOS, ALP, CHOL and AMYL than the SAWIP ($P < 0.05$).

6.3.4 Liver Histometry

Figure 6.1 and Table 6.2 show H & E images and hepatocyte measurements, respectively, of livers of LW x LR crosses and SAWIP fed the CON and HMC diets. The histological analysis of livers from the two breeds on both diets indicated a normal liver lobular architecture. There were no massive infiltrations of inflammatory cells in images of all the treatments and no congestion of sinusoids. Cytoplasmic vacuolization, indicative of fat globules in hepatocytes occurred in images of all the breeds and there were not significant differences among the treatments. The cytoplasm of SAWIP hepatocytes stained more eosinophilic than that of LW x LR crosses on visual inspection. There were breed x diet interactions ($P < 0.05$) for hepatocyte area and concentration. The SAWIP's on the CON diet had smaller mean hepatocyte area but greater number of hepatocytes per unit area than those on the HMC diet while the LW x LR crosses on the CON diet had

greater mean hepatocyte area and less numbers per unit area than those on the HMC diet. There were no differences in hepatocyte area, gray-measure, and concentration of hepatocytes between the diets. Hepatocytes of SAWIP had greater surface area ($P < 0.05$) than those of LW x LR crosses. The hepatocytes of LW x LR crosses had greater ($P < 0.05$) gray-measure than those of SAWIP.

Table 6.1 Serum metabolite measurements in South African Windsnyer-type Indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) finisher pigs fed diets containing ensiled maize at low (LMC) and high (HMC) inclusion levels

Breed		SAWIP			LW x LR				Probability		
Diet		CON	LMC	HMC	CON	LMC	HMC	RSD	Breed	Diet	Breed x Diet
Parameter[1]	Normal ranges										
Metabolic markers											
GLU mg/dL	85-160	77.3^{ł}	72.5^{ł}	62.5^{ł}	86.4*	81.2^{ł}	69.1^{ł}	23.1	0.400	0.433	0.994
CHOL mg/dL	18-79	79.3^{a}	73.0^{a}	87.0^{ab*}	110.6^{b*}	118.1^{b*}	116.1^{b*}	20.6	0.0003	0.800	0.672
Liver function											
TP g/dL	6.0-8.0	7.4	7.7	7.4	7.7	8.3*	7.8	1.39	0.435	0.741	0.978
ALB g/dL	1.8-3.3	4.6*	5*	4.5*	4.6*	4.5*	4.8*	0.67	0.753	0.900	0.436
GLOB g/dL	3.9-6	2.8	3.3	3	3	3.4	3.1	1.22	0.800	0.778	0.990
ALT U/L	9-43	95.0^{b*}	63.3^{a*}	70.0^{ab*}	74.0^{ab*}	76.2^{ab*}	75.9^{ab*}	12.7	0.897	0.094	0.047
A:G		1.8	1.7	1.5	1.6	1.2	1.6	0.573	0.390	0.568	0.762
ALP U/L	92-294	110.3^{ab}	103.8^{a}	133.5^{ab}	163.4^{b}	154.6^{ab}	151.0^{ab}	42.4	0.028	0.814	0.678
TBIL mg/dL	0.1-0.3	0.6*	0.9*	0.5*	0.5*	0.5*	0.6*	0.41	0.490	0.741	0.479
Other serum markers											
PHOS mg/dL	3.6-9.2	9.3^{a*}	12.2^{ab*}	9.3^{a*}	12.7^{b*}	12.3^{b*}	13.3^{b*}	1.91	0.003	0.072	0.417
CAL mg/dL	6.5-11.4	11.6	12.9*	11.3	13.1*	12.9*	12.8*	1.43	0.102	0.464	0.432
CA:P		1.3^{b}	1.1^{ab}	1.2^{ab}	1.0^{ab}	1.0^{a}	1.0^{ab}	0.16	0.012	0.288	0.791
BUN mg/dL	6-30	26.7^{b}	18.8^{ab}	19.0^{ab}	17.8^{ab}	16.3^{a}	15.2^{a}	4.94	0.043	0.254	0.792
CREAT mg/dL	0.5-2.1	0.9^{a}	1.1^{a}	1.2^{ab}	1.6^{b}	1.4^{b}	1.4^{b}	0.246	0.0003	0.778	0.278
AMYL U/L	271-1198	393.3^{a}	612.5^{ab}	798.0^{bc}	862.4^{c}	817.9^{c}	843.7^{c}	147.9	0.001	0.069	0.038

SAWIP n=3; LW x LR n = 6 ; RSD - residual standard deviation
^{a,b} Means with different letters in a row differ significantly ($P<0.05$)
*Denotes values above the normal range; ^{ł}Denotes values below the normal range
[1]Normal ranges
GLU - glucose; BUN - blood urea nitrogen; CREAT - creatinine; PHOS - phosphorus; CAL - calcium; TP - total protein; ALB - albumin; GLOB - globulin; ALP - alkaline phosphatase; CHOL - cholesterol; TBIL - total bilirubin; AMYL - α-amylase ; ALT - alanine aminotransferase

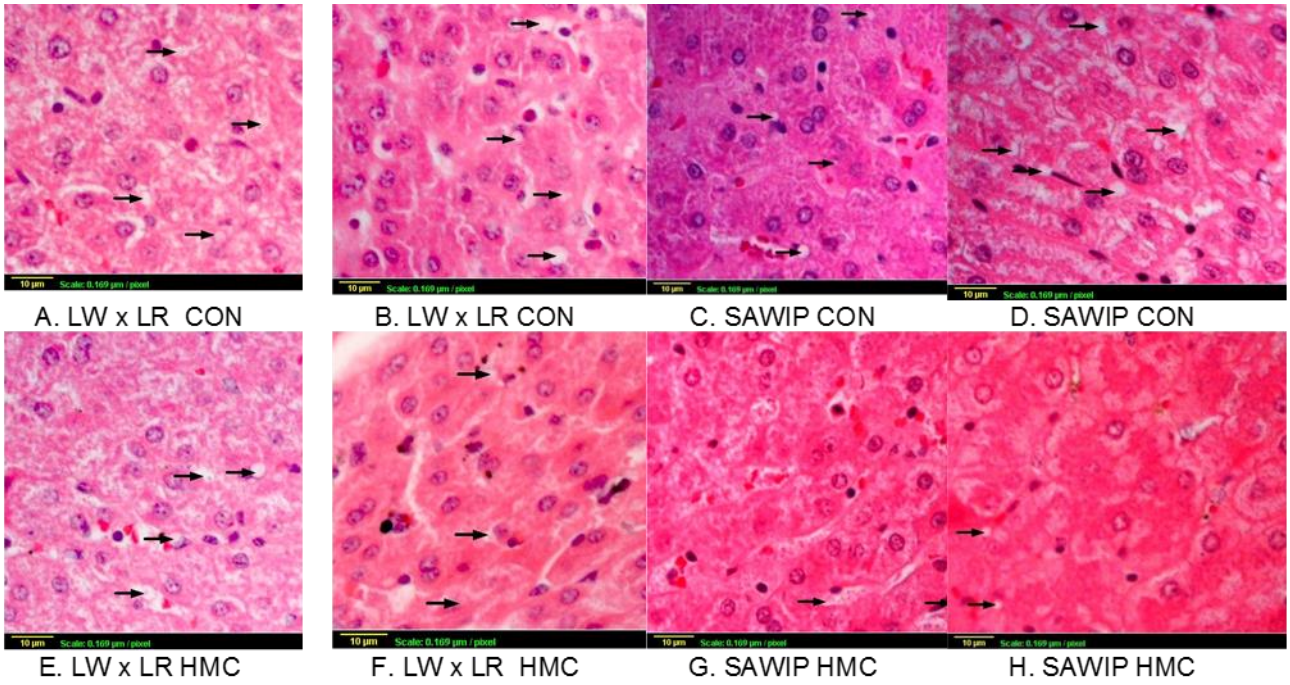

Figure 6.1 Haematoxylin and Eosin images from the livers of Large White x Landrace (LW x LR; n=3) crosses and South African Windsyner-type Indigenous pigs (SAWIP; n=3) fed diets containing no ensiled maize cobs (CON) and high maize cob (HMC) inclusion levels.
Black arrows show cytoplasmic vacuolization. Magnification x40

Table 6.2 Hepatocyte nuclei (HEP) measurements in South African Windsnyer-type Indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) finisher pigs fed diets containing no ensiled maize cobs (CON) and high maize cob (HMC) inclusion levels

Breed	SAWIP		LW x LR			Probability		
Diet	CON	HMC	CON	HMC	RSD	Breed	Diet	Breed x Diet
Parameter[1]								
HEP Area (μm^2)	12.9^a	13.5^b	11.8^c	10.7^d	2.49	<0.0001	0.262	<0.0001
HEP Gray-measure	115.1^a	114.1^a	155.1^b	152.4^b	13.04	<0.0001	0.062	0.586
HEP density (no. per mm^2)	4407^b	3332^a	4050^{ab}	4548^b	1009.7	0.248	0.434	0.039

SAWIP n=3; LW x LR n = 6
^{a,b} Means with different letters in a row differ significantly (P<0.05)
HEP – Hepatocyte nuclei

6.3.5 1D-PAGE

Total proteins extracted from serum and the liver from LW x LR crosses and SAWIP fed CON and HMC diets were firstly separated by one-dimensional (1D)-PAGE. Figures 6.2, 6.5 and 6.8 show the protein patterns for

all the treatments. This confirmed the equality of sample loadings and also showed that the protein band patterns were comparable between the two breeds and two diets.

Protein banding patterns of serum from Large White x Landrace (LW x LR) crosses fed CON and HMC diets and South African Windsyner-type Indigenous pigs (SAWIP) fed high maize cobs diets (HMC) are shown in Figures 6.2, 6.3 and Table 6.3.

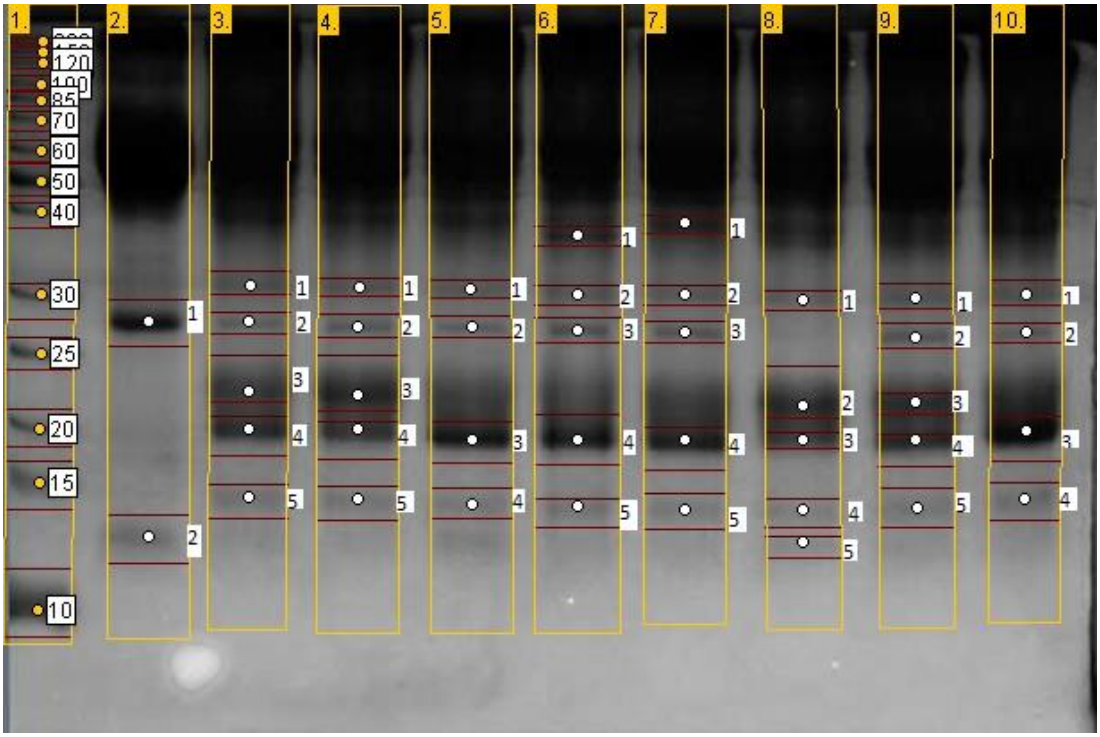

Figure 6.2 Gel image of SDS-PAGE protein banding pattern detected by GelAnalyzer.

SDS-PAGE of albumin depleted serum protein extract profiles of Large White x Landrace (LW x LR) crosses fed control (Lanes 3, 4) and high maize cob level diets (Lanes 5, 6, 7) and South African Windsyner-Type Indigenous pigs fed high maize cob level diet (Lanes 8, 9, 10). Lane 1 = Molecular Marker (kDa); Lane 2 = LW x LR CON containing albumin. Bands in Lane 1 are identified by molecular weight (kDa) and bands in Lanes 2 to10 are identified with arbitrary numbers. More detailed analysis was performed using protein densitometric GelAnalyzer software.

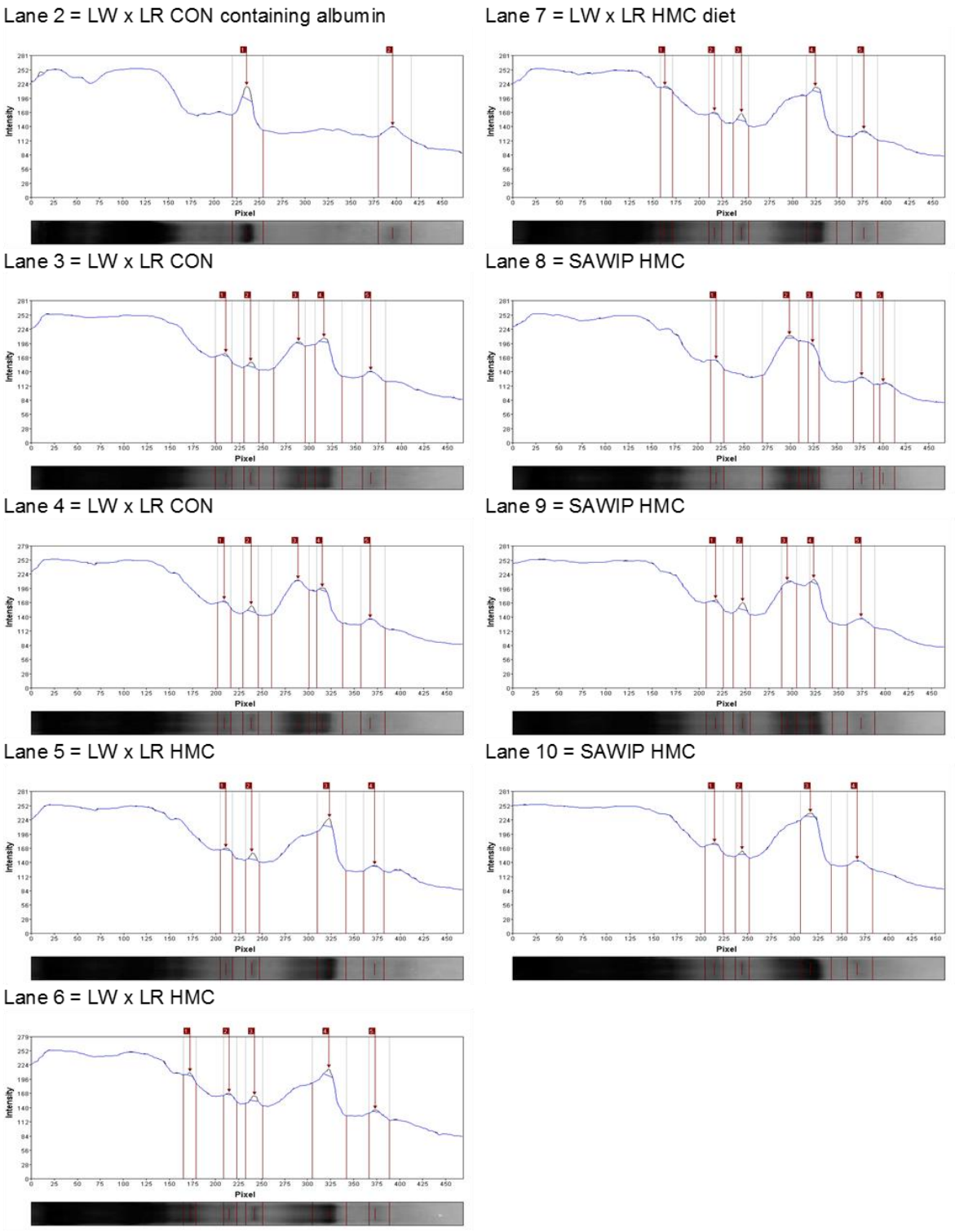

Figure 6.3 Densitometric determination of the intensity and number of bands of albumin depleted serum protein extracts.

Representative lanes of protein bands for the two breeds Large White x Landrace (LW x LR) crosses on control (CON), high maize cob level (HMC) diets, and South African Windsyner-type Indigenous pigs (SAWIP) fed HMC diet were analyzed using GelAnalyzer software.

Table 6.3 Densitometric analysis of albumin depleted serum protein extracts' banding patterns of Large White x Landrace (LW x LR) crosses on control (CON), high maize cob level (HMC) diets, and South African Windsyner-type Indigenous pigs (SAWIP) fed HMC diet.

The table shows the number of bands, the mobility rate (Rf), the amount of the expressed protein bands (Vol) and their molecular weight (MW) for each treatment.

Treatment	Band	Rf	Vol	MW
LW x LR CON	1	0.499	189	24
2 bands	2	0.837	4	22
LW x LR CON	1	0.450	16	25
5 bands	2	0.507	51	24
	3	0.619	13	22
	4	0.677	54	22
	5	0.786	4	22
LW x LR CON	1	0.447	9	25
5 bands	2	0.509	57	24
	3	0.618	6	22
	4	0.673	29	22
	5	0.784	5	22
LW x LR HMC	1	0.451	16	25
4 bands	2	0.511	72	24
	3	0.690	96	22
	4	0.795	7	22
LW x LR HMC	1	0.367	22	29
5 bands	2	0.458	10	25
	3	0.516	58	23
	4	0.689	74	22
	5	0.795	21	22
LW x LR HMC	1	0.352	17	30
4 bands	2	0.467	13	25
	3	0.529	80	23
	4	0.700	56	22
	5	0.812	16	22
SAWIP HMC	1	0.471	2	24
5 bands	2	0.640	27	22
	3	0.694	7	22
	4	0.807	7	22
	5	0.857	12	22
SAWIP HMC	1	0.470	16	24
5 bands	2	0.532	84	23
	3	0.636	9	22
	4	0.696	30	22
	5	0.806	3	22
SAWIP HMC	1	0.467	14	25
4 bands	2	0.530	28	23
	3	0.689	43	22
	4	0.798	4	22

The 24 kDa molecular weight (MW) protein band was observed more consistently in the LW x LR on the CON diets and in SAWIP on the HMC diet than in the LW x LR on the HMC diet (Table 6.3; Band 2 in Lanes 3 and 4; vs Band 1 in lanes 8 and 9 in Figure 6.2). The protein bands however differed in their mobility rates (Rf values; Table 6.4; 508 for the LW x LR vs 470 for the SAWIP) and in the intensity of their expression ($P < 0.05$) (Figure 6.4). A protein band (MW 25 kDa) was observed in LW x LR crosses on CON and HMC diets but was absent in the SAWIP on the HMC diet. There was no difference in band intensity of 22 kDa protein bands between the LW x LR CON and LW x LR HMC diet. Intensity of 22 kDa protein bands in LW x LR on the HMC diets was greater ($P < 0.05$) than in SAWIP on HMC diet (Figure 6.4).

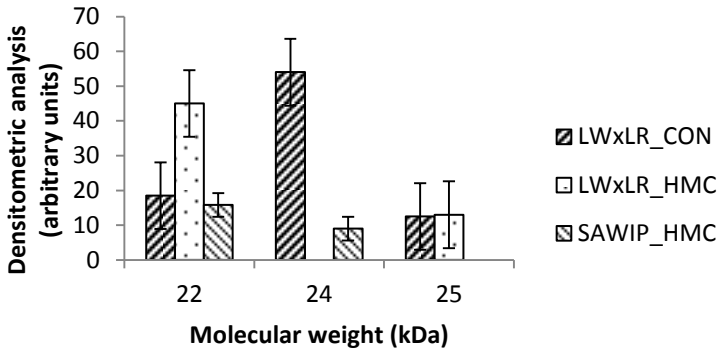

Figure 6.4 Albumin depleted serum protein extracts' bands' intensity of 22, 24 and 25 kDa molecular weight (MW) from Large White x Landrace (LW x LR) crosses on control (CON), high maize cob level (HMC) diets, and South African Windsyner-type Indigenous pigs (SAWIP) fed HMC diet quantified using GelAnalyzer

Protein band profiles of serum from SAWIP fed CON and HMC diets LW x LR crosses fed HMC are shown in Figures 6.5, 6.6 and Table 6.4. Four protein bands of molecular weights 21, 22, 24 and 26 kDa (Table 6.4) were observed consistently in the SAWIP on the CON and HMC diets and in LW x LR crosses on the HMC diet. There were no differences in intensity of the protein bands of 21, 22, 24 and 26 kDa MW between the breeds and diets (Figure 6.7). Protein bands of MW 22 kDa were present in SAWIP on CON and HMC diets and absent from LW x LR on HMC diets. Protein bands of MW 36 kDa were present in SAWIP on HMC diets and absent from LW x LR on HMC diets.

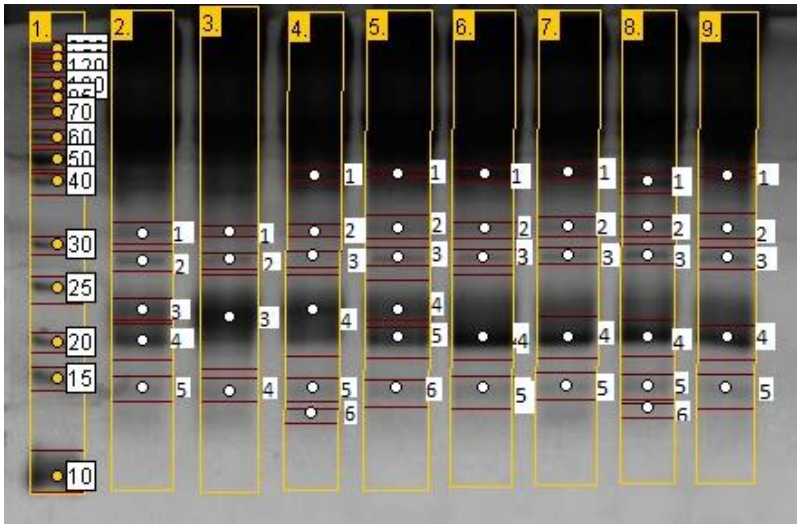

Figure 6.5 Gel image of SDS-PAGE Protein banding pattern detected by GelAnalyzer.

SDS-PAGE of albumin depleted serum protein extracts' profiles in serum of South African Windsyner-type Indigenous pigs fed control (Lanes 2, 3) and high maize cob level diets (Lanes 4, 5, 6) and Large White x Landrace (LW x LR) crosses fed high maize cob level diet (Lanes 7, 8, 9). Lane 1 = Molecular Marker (kDa); Bands in Lane 1 are identified by molecular weight (kDa) and bands in Lanes 2 to 9 are identified with numbers. More detailed analysis was performed using protein densitometric GelAnalyzer software.

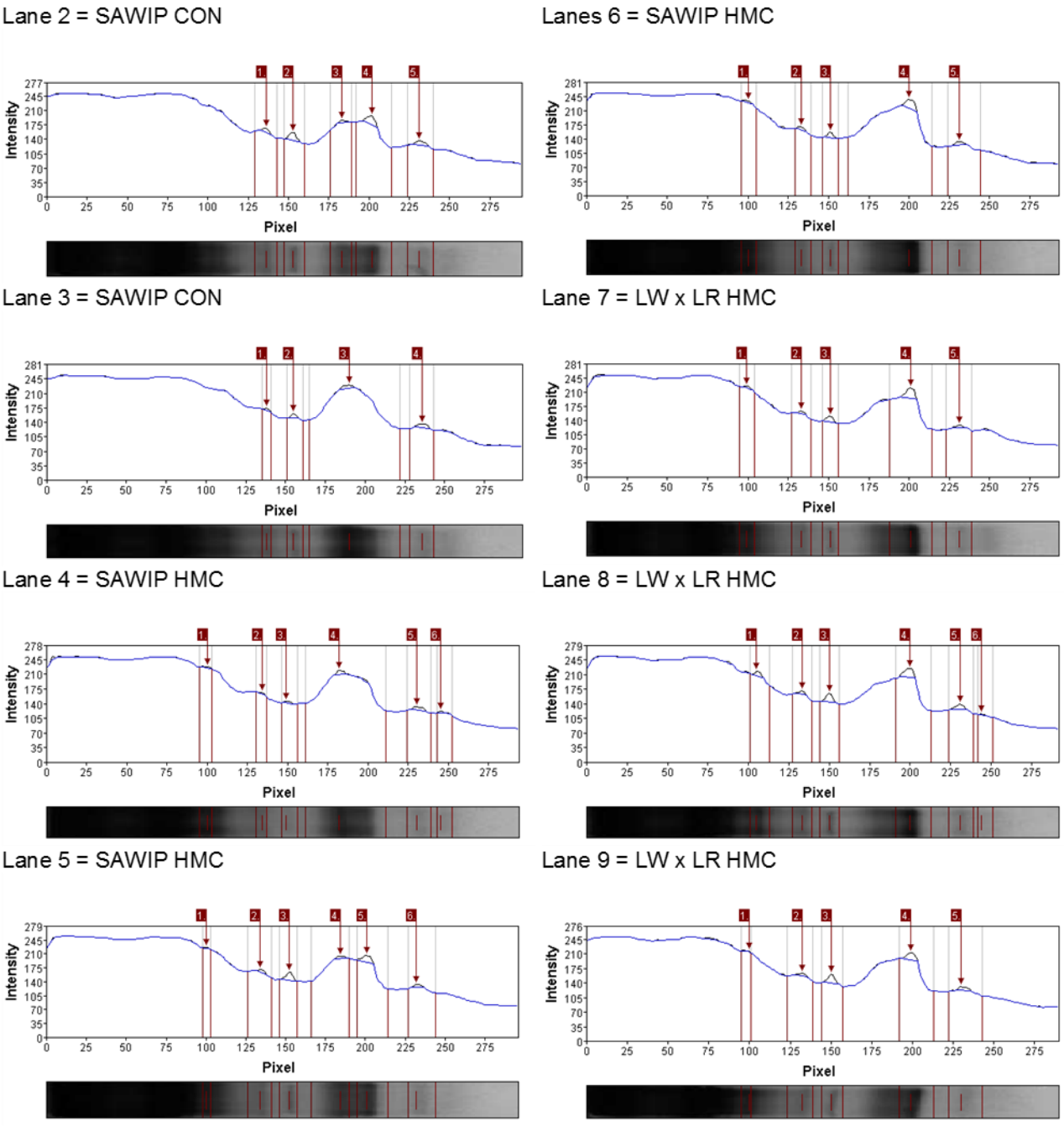

Figure 6.6 Densitometric determination of the intensity and number of bands of albumin depleted serum protein extracts.

Representative lanes of serum protein bands for the two breeds South African Windsyner-Type Indigenous pigs (SAWIP) on control (CON), high maize cob level (HMC) diets, and Large White x Landrace crosses (LW x LR) fed HMC diet were analyzed using GelAnalyzer software.

Table 6.4 Densitometric analysis of albumin depleted serum protein extracts' banding patterns from South African Windsyner-type Indigenous pigs (SAWIP) on control (CON), high maize cob level (HMC) diets, and Large White x Landrace (LW x LR) crosses fed HMC diet.
The table shows the number of bands, the mobility rate (Rf), the amount of the expressed protein bands (Vol) and the molecular weight (MW) for each band

Treatment	Band	Rf	Vol	MW
SAWIP CON	1	0.461	38	26
5 Bands	2	0.519	101	24
	3	0.62	24	22
	4	0.685	113	21
	5	0.783	78	21
SAWIP CON	1	0.462	15	26
4 bands	2	0.518	37	24
	3	0.635	57	22
	4	0.789	55	21
SAWIP HMC	1	0.34	11	36
6 bands	2	0.456	9	26
	3	0.507	18	24
	4	0.619	58	22
	5	0.782	45	21
	6	0.833	12	21
SAWIP HMC	1	0.338	9	36
6 bands	2	0.453	29	26
	3	0.514	94	24
	4	0.622	28	22
	5	0.679	102	21
	6	0.784	35	21
SAWIP HMC	1	0.341	22	36
5 bands	2	0.454	25	26
	3	0.515	51	24
	4	0.683	151	21
	5	0.788	44	21
LW x LR HMC	1	0.338	19	36
4 bands	2	0.454	27	26
	3	0.515	66	24
	4	0.686	156	21
	5	0.788	31	21
LW x LR HMC	1	0.36	43	33
4 bands	2	0.455	30	26
	3	0.514	95	24
	4	0.685	137	21
	5	0.791	71	21
	6	0.836	5	21
LW x LR HMC	1	0.345	5	35
5 bands	2	0.455	35	26
	3	0.517	97	24
	4	0.686	93	21
	5	0.793	47	21

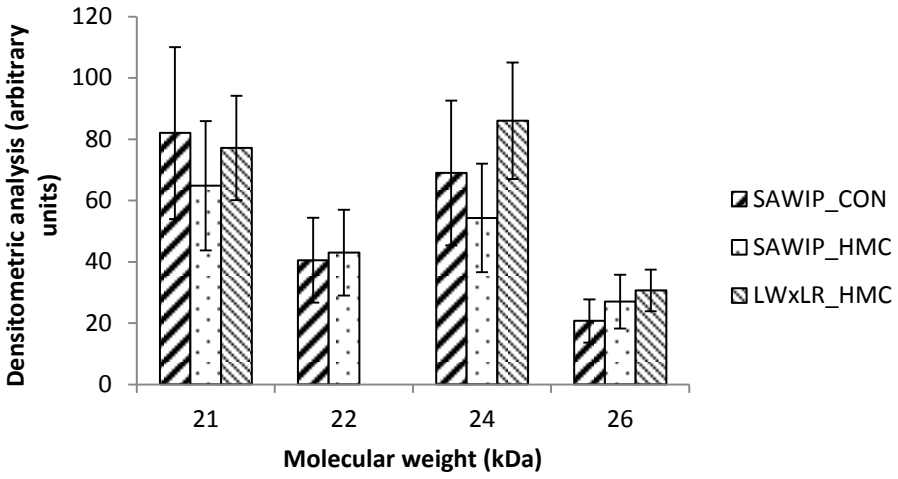

Figure 6.7 Albumin depleted serum protein extracts' bands' intensities of 22, 22, 24 and 26 kDa molecular weight (MW) from Large South African Windsyner-type Indigenous pigs (SAWIP) on control (CON), high maize cob level (HMC) diets, and White x Landrace (LW x LR) crosses fed HMC diet quantified using GelAnalyzer

Protein profiles from liver extracts of LW x LR crosses and SAWIP fed high maize cobs diets are in Figures 6.8, 6.9, 6.10 and Table 6.5. The 20 µg concentration produced on average 7.5 distinct protein bands while the 30 µg SAWIP had on average 3 distinct protein bands as shown in Figure 6.8 and Table 6.6. There were no protein bands of similar molecular weight across the two breeds except for the 25 kDa MW band which was present in both.

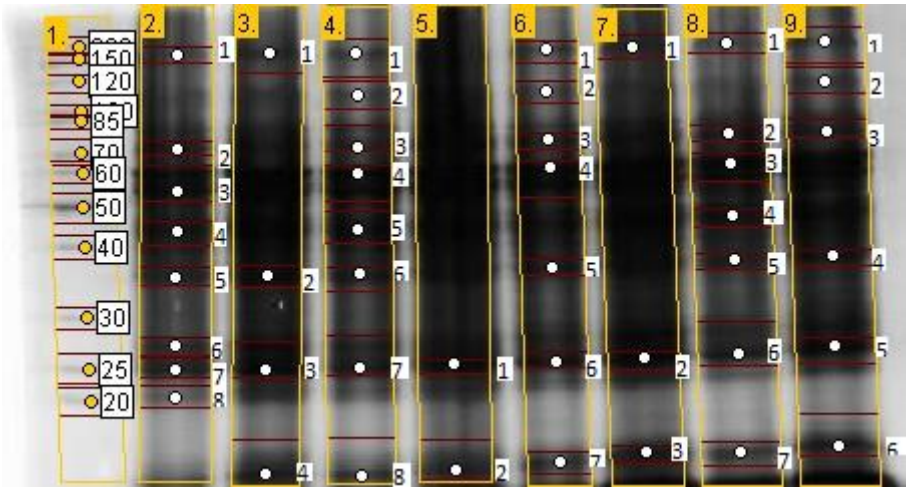

Figure 6.8 SDS-PAGE Gel image of liver protein extracts' banding patterns detected by GelAnalyzer software.

Banding patterns of South African Windsyner-type Indigenous pigs and Large White x Landrace (LW x LR) crosses fed a high maize cob level diet were compared at two protein concentrations; 20 vs 30 µg. Lanes 2 and 4 = LW x LR at 20 µg; Lanes 3 and 5 = LW x LR at 30 µg; Lanes 6 and 8 = SAWIP at 20 µg; Lanes 7 and 9 = SAWIP at 30 µg. Bands in Lane 1 are identified by molecular weight (kDa) and bands in Lanes 2 to 9 are identified with numbers. Detailed analysis was performed using protein densitometric GelAnalyzer software.

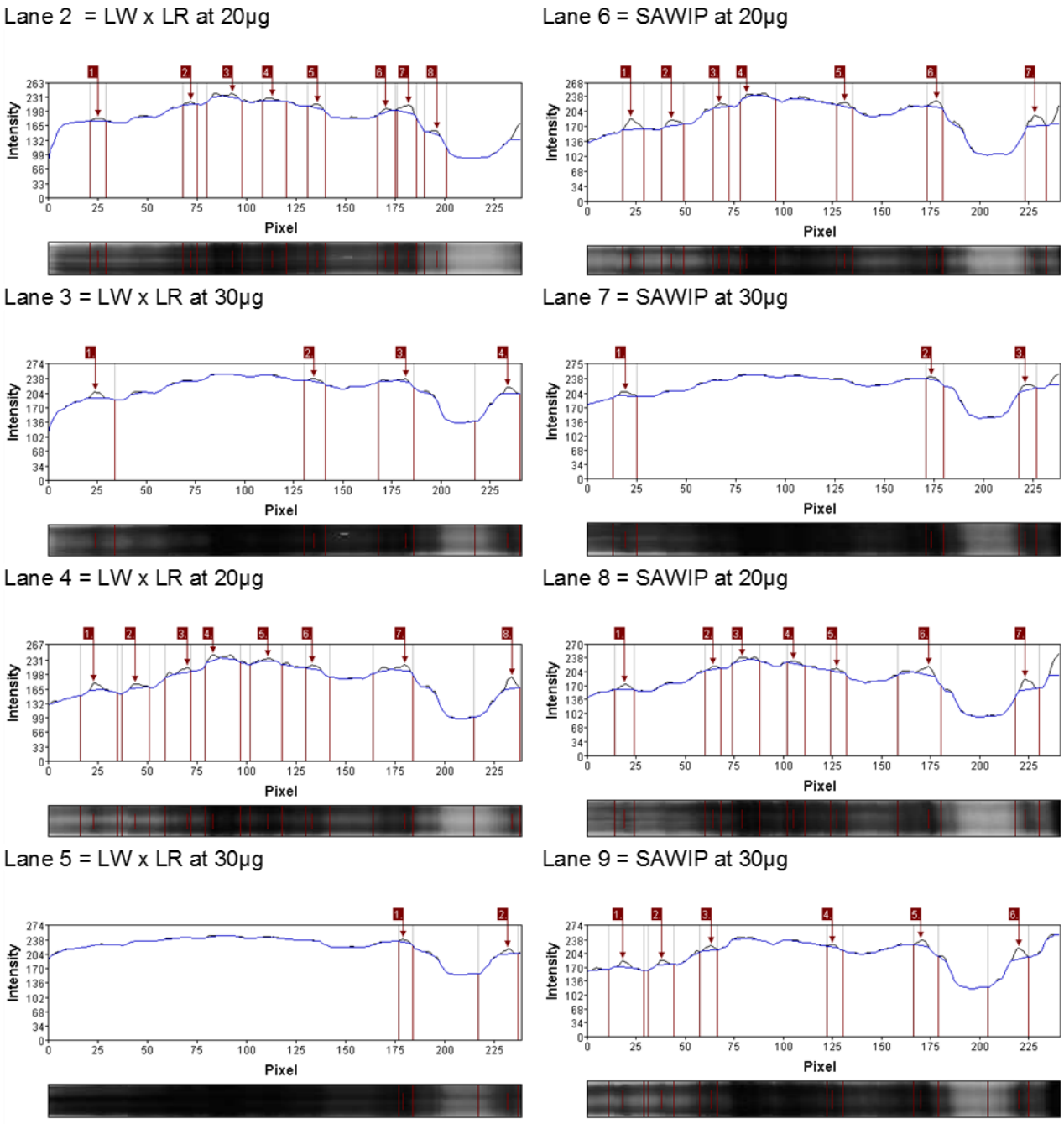

Figure 6.9 Densitometric determination of the intensity and number of bands of liver protein extracts.

Representative lanes of liver protein bands for the two breeds South African Windsyner-type Indigenous pigs (SAWIP) and Large White x Landrace crosses (LW x LR) fed high maize cob level (HMC) diets analyzed using GelAnalyzer software.

Table 6.5 Densitometric analysis of liver protein extracts' banding patterns from South African Windsyner-type Indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) crosses fed high maize cob level (HMC) diets.
The table shows the number of bands, the mobility rate (Rf), the amount of the expressed protein bands (Vol) and their molecular weight (MW) for each breed and protein concentration.

Treatment	Band	Rf	Vol	MW	Treatment	Band	Rf	Vol	MW
LW x LR at 20µg	1	0.105	34	151	LW x LR at 30µg	1	0.749	30	27
8 bands	2	0.301	20	64	2 bands	2	0.971	46	25
	3	0.389	58	48	SAWIP at 20µg	1	0.091	146	162
	4	0.473	35	39	7 bands	2	0.178	77	107
	5	0.569	38	32		3	0.278	41	70
	6	0.711	31	28		4	0.336	46	57
	7	0.762	109	27		5	0.544	46	34
	8	0.82	24	26		6	0.739	68	27
LW x LR at 30µg	1	0.1	79	155		7	0.946	139	25
4 bands	2	0.56	33	33	SAWIP at 30µg	1	0.079	65	172
	3	0.755	35	27	3 bands	2	0.728	35	28
	4	0.971	77	25		3	0.925	82	25
LW x LR at 20µg	1	0.096	84	158	SAWIP at 20µg	1	0.079	65	172
8 bands	2	0.184	56	104	7 bands	2	0.266	25	73
	3	0.293	72	66		3	0.328	69	58
	4	0.347	85	55		4	0.436	31	42
	5	0.464	37	39		5	0.527	28	35
	6	0.556	43	33		6	0.722	134	28
	7	0.753	101	27		7	0.925	133	25
	8	0.979	128	25	SAWIP at 30µg	1	0.075	74	176
					6 bands	2	0.158	44	117
						3	0.261	58	75
						4	0.519	25	35
						5	0.705	65	28
						6	0.913	141	25

6.3.6 2D-PAGE Liver proteome patterns

The 2D gels of liver protein bands from LW x LR crosses and SAWIP fed high maize cobs diets separated by pH 3-10 in the first dimension and 12 % SDS PAGE in the second dimension are shown in Figure 6.8. The liver protein patterns observed for the two breeds were similar as shown in the 2D gel images in Figure 6.8. Over 40 spots could be seen on the gels but resolution of most of the protein spots was poor indicating that the pH- range chosen was not appropriate. Most of the protein spots lay in the range pH 4-7. Of the resolved protein spots, four Coomassie stained protein spots that showed the most pronounced differences between the two breeds were isolated, digested with trypsin, analyzed by PMF and are presented in Table 6.6.

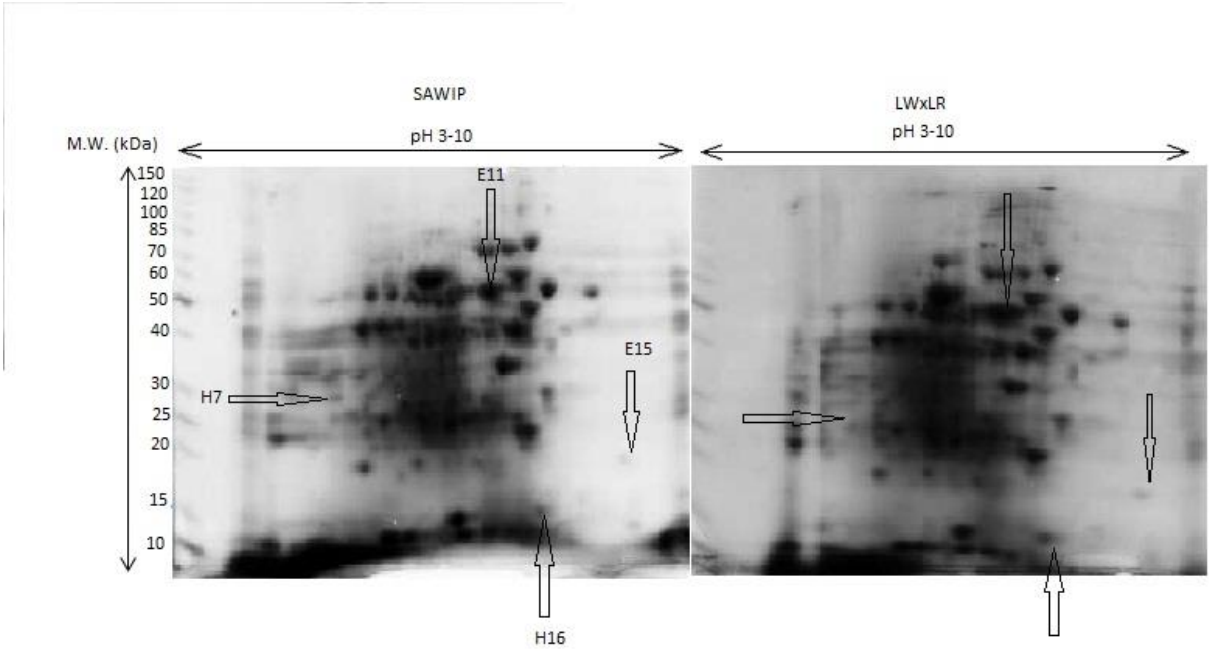

Figure 6.8 2D-PAGE gel images of liver protein extracts of Large White x Landrace (LW x LR) crosses and South African Windsyner-type Indigenous pigs (SAWIP) fed high maize cobs diets run at pH range 3-10

6.3.7 Protein identity

In order to identify the differentially expressed proteins, the protein spots were excised from the gels and analyzed by MALDI-TOF and MS/MS analysis. Two of the protein spots identified; Beta-Casein (H16) and Chain A, Murine Pebp-2 (Phosphatidylethanolamine-Binding Protein-2) (E15) were not pig specific and had a low number of peptides matched; 1 and 2 respectively. Gene Ontology (GO) search showed that Guanidinoacetate N-methyltransferase-like isoform 1 (spot H7) is involved in creatine biosynthetic processes; and Catalase (spot E11) is involved in hydrogen peroxide catabolic processes, aerobic respiration and cholesterol metabolic processes.

Table 6.6 Hepatic proteins differentially expressed in Large White x Landrace (LW x LR) crosses and South African Windsyner-type Indigenous pigs (SAWIP) fed high maize cobs diets

Spot No.	Name of protein[1]	Accession no.	Sequence Coverage (%)	MW (kDa)/pI	Species	MOWSE Score	Database	No. of peptides matched
E11	Catalase	gil356460899	15.7	59.9/6.65	Sus scrofa	257.16	NCBInr	6
H16	Beta-casein	gil49781319	19.4	11.1/7.71	Capra hircus	138.08	NCBInr	2
H7	Guanidinoacetate N-methyltransferase-like isoform 1	gil335282287	23.7	26.5/5.24	Sus scrofa	127.51	NCBInr	6
E15	Chain A, Murine Pebp-2 (Phosphatidylethanolamine-Binding Protein-2)	gil21730513	10.9	20.7/9.36	-	50.60	NCBInr	1

[1]All proteins were upregulated in the LW x LR crosses

6.4.3 Liver histometry

The study compared changes in serum metabolite profiles and liver morphology, and also liver and serum protein changes in LW x LR and SAWIP pigs fed a diet containing high levels of ensiled maize cobs. The liver morphology was evaluated in H & E stained images and there were no visible anomalies that could be attributed to the high fibre diets. Hematoxylin, a basic dye, colors ribosomes and the chromatin-rich cell nucleus blue-purple; and eosin an alcohol-based acidic dye, colors intracellular or extracellular proteins bright pink (Amin & Mahmoud-Ghoneim, 2011). The more intense eosinophilic staining in SAWIP H & E images was indicative of more proteinacious material in the liver of this breed. The hepatocyte parameters for the two breeds differed for the CON and HMC diets suggesting differences in how the two breeds adapted to HMC diets. The higher gray-measure value in LW x LR liver suggests that they had a greater concentration of ribosomes and chromatin in the cell nucleus compared to the SAWIP.

6.4.4 Blood metabolite responses

6.4.4.1 Metabolic markers

Pigs on a high fibre diet are likely to have high CHOL levels from increasing availability and absorption of acetate and a high GLU concentration resulting from increased availability of sugars from the fermentation of fibre (Ziemer et *al*., 2012). The GLU levels in the current study were below the normal range and the responses of LW x LR and SAWIP in regulating the GLU levels were similar. This corroborates findings by Mashatise *et al*. (2005) who reported no differences in plasma GLU in Mukota and Mukota x Large White gilts fed a control and a high fibre diet with 20 % maize cobs. In contrast to these findings however, Pond *et al*. (1980) reported lower GLU levels in obese pigs than in lean or contemporary pigs. As maize cob level in the diet increased, there was no change in GLU levels, in contrast to the results of Frank *et al*. (1983) who reported a decrease in GLU levels as maize cob levels increased.

Since the SAWIP tend to lay fat easily they should, in theory have higher serum CHOL levels. Nonetheless, CHOL levels in the LW x LR were higher than both the normal range and those of the SAWIP. These results are in contrast to those of Ferna'ndez-Fi'gares *et al*. (2007) who found no differences in CHOL serum levels from Iberian (an obese genotype) and Landrace (a lean genotype) gilts. Mersmann *et al*. (1982) also did not find differences between lean and obese pigs in serum lipids. Pond *et al*. (1986) however, reported that plasma CHOL increased dramatically in lean pigs fed low protein diets even though feed intake and body weight gain were less and were more adversely affected by diet than in obese pigs. Ferna'ndez-Fi'gares *et al*. (2007)'s reports of an inverse relationship between dietary protein levels and CHOL agree with these findings. By implication, the protein levels in LW x LR may have been limiting in this study or much acetate was being produced in the intestines and metabolized to CHOL to which it is a precursor.

6.4.4.2 Markers of liver health and function

Total protein was within the normal ranges suggesting that dietary protein intake and the liver synthetic function on the diets containing ensiled maize cobs was adequate (Pond *et al*., 1986). The higher than normal levels of serum albumin found in the pigs on all treatments are possibly a reflection of dehydration (Kaneko, 1989). However, there is no clear explanation for the dehydration because all the pigs were rested before slaughter, had access to water in the lairages and had been transported for only about fifteen minutes to the abattoir. Since other metabolites like TP, CHOL, ALT and TBIL were also either higher than or at the upper limit of the normal ranges, dehydration is a plausible explanation. A definitive diagnosis of dehydration would have been made by determination of the plasma osmolarity, but this could not be done owing to technical limitations. Different responses in ALT levels in the LW x LR and SAWIP were induced by LMC and HMC diets. The ALT concentrations in the SAWIP on the CON diet were higher than other treatments. Levels of ALT increase mainly due to hepatocellular and less to muscle damages (Varghese *et al*., 2012). The possibility of increases in ALT being a result of muscle damage are minimal because all the pigs were handled uniformly, no fighting was observed and pigs from different treatments were slaughtered in a random sequence. Haemolysis of samples can however affect ALT levels as well as other parameters in clinical biochemistry measurements (Koseoglu *et al*., 2011). There was no inflammatory response in the liver given that there were no changes in total serum protein and in the albumin: globulin ratio and no inflammatory cells in the H & E livers images. An inflammatory disease state frequently results in increasing protein globulin fraction, which was not the case in this study. A possible explanation could lie in the elevated TBIL levels in all the treatments, which were at least twice above the normal range. Total bilirubin levels are elevated when there is obstruction of the bile duct system or severe hepatic disease (Kaneko, 1989). Additional tests would be needed to get a clearer picture of the causes.

6.4.4.3 Other serum markers

Chivandi *et al*. (2006) suggested that serum α-amylase should be an indicator of pancreatitis or salivary gland pathology such as inflammation. Serum α-amylase in animals comes from the pancreas and other extra-pancreatic sources such as salivary glands and duodenal mucosal cells in pigs (Duncan *et al*., 1996). Serum α-amylase also increased with severe enteritis (Zantop, 1997) and was influenced by levels of dietary phytase and phytate in chickens (Liu *et al*., 2008). However, since the AMYL levels were within the normal range; the observed differences between the breeds, and the dietary related differences in the SAWIP as the level of maize cobs were increased could be an indication of an adaptation inherent in the SAWIP to cope with the diets containing ensiled maize cobs.

There is no clear explanation for the higher than normal CAL and PHOS levels in all the treatments. It could have been due to increased availability of the minerals because of improved digestibility of the diets. Tietz (1982) reported a reciprocal relationship between CAL and PHOS levels and Prikoszovits & Schuh (1995) reported CAL:PHOS ratios in serum of 0.94 +/- 0.16 in fattening pigs, similar to what was obtained in the study. Creatinine levels were in the normal ranges for both breeds and in all diets, but the LW x LR had

higher levels than the SAWIP. Creatinine is a product of creatine phosphate metabolism. It is an energy substrate used by muscle and is a biomarker for renal function evaluation along with BUN (Faulkner & King, 1982). Creatinine is released from muscle in amounts proportional to muscle mass (Rassin & Bhatia, 1992; Ferna'ndez-Fi'gares *et al*., 2007). Higher CREAT levels in the LW x LR therefore relate to their increased lean mass. Urea, a principal product of the catabolism of protein and the levels of BUN can act as indicators of body protein status (Kohn *et al*., 2005) and efficiency of renal function. The BUN levels for pigs on all treatments in this study were within the normal range and only the SAWIP pigs on the CON diet had higher levels than on all other treatments. Blood urea nitrogen levels increase with dietary increases in protein. Since BUN levels have been used to determine protein requirements and lean tissue growth rates in pigs (Chen *et al*., 1995; Coma *et al*., 1995) results from the current study could be used to make dietary recommendations for the SAWIP. Frank *et al*. (1983) on the other hand reported an increase in urea levels in crossbred pigs fed diets with incremental levels of 7.5 and 15 % maize cobs and attributed it to increased ammonia production by intestinal microbes. Berschauer *et al*. (1983) reported that a reduction in BUN levels is associated with an increase in the efficiency of nitrogen utilization. This suggests that the increase in ensiled maize cobs in the diet in the SAWIP improved the efficiency of nitrogen utilization.

6.4.5 Proteomic analyses

The approach taken for proteomic analyses was to establish a proof of principle that feeding high fibre diets would influence changes in serum and liver protein extracts in the two breeds. Towards this end 1D SDS-PAGE's attractive features, including excellent mass resolution, superior protein solubilization, accommodation of large amounts of protein, and good run-to-run reproducibility made it a logical and critical first step (Ahmad *et al*., 2005). Protein band patterns of serum from LW x LR crosses and SAWIP fed CON and HMC diets showed differences related to breed and diet. The difference in intensity of 22 kDa protein bands between LW x LR and SAWIP on the HMC diets could be an indication of breed differences in responses to high fibre diets. The Coomassie staining intensity of 36 kDa protein bands observed in SAWIP on the HMC diet suggests potential of the presence of biomarkers of fibre utilisation. The 1D gels were not reproducible for the serum samples highlighting the limitations of 1D for the analysis of complex protein samples. Nevertheless, the 1D gels also clearly show that there are differences, warranting the use of a time-consuming and expensive method like 2DE for further analysis.

Unlike the 1D, the 2D SDS-PAGE can resolve up to thousands of protein spots facilitating their identification. Even though only a few gels were run, they were deemed sufficient to establish the proof of principle. In the 2D SDS-PAGE analysis, an IPG strip with pH range 3-10 was used in order to visualize proteins over a wide range of pIs. While the chosen pH range gave an overview of where most of the proteins were situated on the pH gradient, future work will use an IPG strip of the range pH 4-7 to get better resolution of protein spots. The lack of resolution implies that it is very likely that there was more than one protein in each spot subjected to MALDI-MS, which hindered identification by PMF. This explains unexpected results like a hit for goat casein Beta-casein and Pebp-2 (Phosphatidylethanolamine-Binding Protein-2). Importantly, despite the

technical issues with this first experiment, some of the proteins identified through MS can explain differences observed in the blood metabolites. For example Guanidinoacetate N-methyltransferase-like isoform 1, is involved in the metabolism of creatine, which was elevated in LW x LR serum. Catalase identified in a spot upregulated in LW x LR is involved in cholesterol metabolic processes, of which matches the observation of elevated cholesterol in the LW x LR in the serum parameter analysis.

6.5 Conclusions

The study demonstrated that ALT and AMYL levels in LW x LR and SAWIP respond differently to diets containing maize cobs. There were also differences in serum and liver proteins and in serum metabolite levels that were diet and breed related. This suggests that proteomics can play a role in evaluating pigs fed diets containing high fibre levels at molecular level. The study showed that protein levels provided in the diet were higher than SAWIP's requirements. A proof of principle to assess serum and liver protein profiles of pigs fed a a high fibre diet using a sodium dodecyl sulphate polyacrylamide gel electrophoresis matrix-assisted laser desorption ionization mass spectrometry (SDS-PAGE /MALDI MS) workflow was established.

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Chapter 7

7 Growth performance and carcass characteristics of growing and finishing South African Windsnyer-type Indigenous and Large White x Landrace crossbred pigs fed on diets containing ensiled maize cobs

Abstract

This study compared breed effects on growth performance and carcass characteristics when ensiled maize cobs were included in pig diets. Fifty Large White × Landrace crossbred pigs (LW x LR) and 30 South African Windsnyer-type Indigenous pigs (SAWIP) were assessed. They consumed a control diet (CON), a low inclusion of ensiled maize cob (LMC) and a high inclusion of ensiled maize cob (HMC) diet in a completely randomized block design. The LW x LR crosses had greater ($P < 0.05$) final weight (FW), average daily feed intake (ADFI), dry matter intake (DMI), average daily gain (ADG) and growth to feed (G:F) ratios than the SAWIP at both the grower and finisher stages. The SAWIP consumed more feed per metabolic body weight ($BW^{0.75}$) than LW x LR crosses at grower stage ($P < 0.05$). There were breed x diet interactions ($P < 0.05$) for P2 measurements and drip loss (DL) in the finishers. The LW x LR growers and finishers had greater ($P < 0.05$) warm and cold carcass weights (WCW, CCW), carcass length (CL), DL, pH_{24}, eye muscle area (EMA) and Lean % than those of SAWIP growers and finishers. The LW x LR finishers on the CON diet had greater ($P < 0.05$) WCW and CCW than those on the HMC and LMC diets. There were diet x breed interactions for dorsal fat thickness at first rib (DFT1), dorsal fat thickness at last lumbar vertebra (DFT3), backfat thickness (BFT) and hind quarter weight proportion (HQWP) in the growers. The dorsal fat thickness at last rib (DFT2) and DFT3 measurements of SAWIP growers on CON and LMC diets were greater ($P < 0.05$) than those on the HMC diet. The breed of pig influenced most of the growth performance and carcass parameters more than the diet did. The SAWIP demonstrated an adaptation to high fibre diets by consuming more feed than the LW x LR per $BW^{0.75}$ at the grower stage. Since the inclusion of ensiled maize cobs in diets did not affect negatively the selected important commercial pork cuts in South Africa, this implies that maize cobs are essential as pig feed

Keywords: fermentation, pig genotypes, fibre, serum enzymes, blood metabolites

7.1 Introduction

Maize cobs have been incorporated at different inclusion levels in pig diets in efforts to offset high feed costs (Ndindana *et al*., 2002; Kanengoni *et al*., 2004; Ndubuisi *et al*., 2008). However, increasing the inclusion levels led to a reduction in average daily gain and feed intake (Frank *et al*., 1983; Kanengoni *et al*., 2004; Ndubuisi *et al*., 2008). This was attributed to the high fibre content of the maize cobs (930 g NDF /kg DM; 573 g ADF/kg DM) which is often lignified by the time of harvest (Kanengoni *et al*., 2002). Frank *et al*. (1983) ranked pigs into high, medium and low performers based on ability to utilize maize cob diets and suggested that genetic and physiological determinants of feed intake influence responses to the maize cob diets. Mukota pigs, an indigenous pig from Zimbabwe utilized diets containing maize cobs better than the Large White and Landrace (LW x LR) crosses (Ndindana *et al*., 2002; Kanengoni *et al*., 2004). Sub-Saharan Africa has large populations of indigenous breeds similar to the Mukota on smallholder farms, that could benefit from maize cob based diets (Chimonyo *et al*., 2005) among which is the South African Windsnyer-type Indigenous pigs (SAWIP). Maize cobs can also benefit the mainstream pig industry since mature commercial pigs can utilize high fibre diets and the incorporation of maize cobs is a potentially economically viable proposition. Results from previous chapters showed an increase in digestibility of nutrients and differences in preferences, microbiome and proteomic expression when ensiled maize cobs were included in pig diets. The objective of the current study was therefore to evaluate growth performance, and carcass characteristics of indigenous and commercial pigs fed ensiled maize cobs incorporated to diets in different proportions.

7.2 Materials and methods

7.2.1 Ensiling process and diets

Maize cobs (920 g/kg DM) were collected from the Agricultural Research Council - Animal Production Institute fields (ARC-API, Irene, Gauteng, South Africa), ensiled and diets formulated as described in Chapter 4, Section 4.2.1. The diets were formulated to provide 14 MJ/kg digestible energy (DE), 180 g crude protein (CP)/kg and 11.6 g lysine /kg which meet and exceed the requirements of growing pigs (NRC, 1998). However, diets could not be formulated to meet the specific SAWIP's nutritional requirements because they are largely unknown. The three diets shown in Table 4.2 were; CON (control diet without maize cobs), LMC (diet containing 100 g maize cobs·kg^{-1} diet, as fed), HMC (diet containing 200 g maize cobs·kg^{-1}diet, as fed). Diets were prepared in quantities sufficient to feed pigs for a week in order to prevent spoilage. The effect of these diets on growth performance, and carcass characteristics in growing pigs were evaluated. The experimental procedures described in this study were approved by the Animal Ethics Committee of the Agricultural Research Council, Animal Production Institute (ARC-API).

7.2.2 Pigs, housing and experimental design

Fifty LW x LR crossbred pigs weighing 28 ± 3.2 (±SD) kg live weight and thirty SAWIP weighing 15 ± 3.2 (±SD) kg, balanced on sex were randomly selected from the ARC-Irene pig breeding units and used in the growth study. Each of the LMC and HMC diets were allocated 18 LW x LR pigs (9 males and 9 females) while the CON had 14 (7 males and 7 females). Each diet was allocated 10 SAWIP (5 males and 5 females). As the LW x LR and SAWIP differ in mature weight (300 to 350 kg vs 140 to 180 kg respectively), growing pigs at the physiological age of proportionately about 0·1 of their mature body weights were selected from each breed as done in a previous study (Kanengoni *et al*., 2004). The LW x LR were housed in 2 x 1.5 m pens and the SAWIP were in 1.5 x 0.9 m pens in separate facilities in environmentally controlled houses with the temperature ranging from 22 to 25 °C as stated in Chapter 4. Pigs were housed individually and the feeders were checked and adjusted twice each day to ensure constant access to fresh feed and minimize any possible wastage. The experimental design was a completely randomised block design. Water was freely available through nipple drinkers. Half the number of pigs in each breed (25 LW x LR and 15 SAWIP) was slaughtered after 28 d as growers (GRO) while the remainder of the pigs were slaughtered after 56 d as finishers (FIN) for the LW x LR and 75 d for the SAWIP FIN.

7.2.3 Measurements

Pigs were weighed individually at the start and weekly until termination. The pigs had free access to feed and feed intake was measured daily by subtracting refusals. Refusals were not analysed but they were visually examined to see if the pigs were selecting against maize cobs. Selection was high in the first week after which the pigs consumed all feed offered. Average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated over the period each group of animals were in the trial from the body weight and feed intake values. After 28, 56 and 75 days, pigs were slaughtered at an abattoir located close-by at 0800 for carcass measurements. Pig carcasses were processed according to the routine abattoir procedures which included an ante-mortem inspection and rest for the pigs before slaughter. The pigs were then stunned with an electrical stunner set at 220 V and 1.8 A with a current flow for 6 s and exsanguinated within 10 s of stunning.

Dehairing and evisceration were done according to the abattoir's standard operating procedures. Warm carcass weight (WCW) was measured after dressing using an overhead scale. Dressing percentage (DP) calculations were determined as the warm carcass weight as a percentage of live weight. The carcasses were then placed in a cold room, kept at an approximate temperature of 0 ºC for 24 hours after which cold carcass weights (CCW) were measured. At 24 h after slaughter, pH_{24} and temperature readings were taken from the *Longissimus thoracis* muscle (eye muscle) with a portable pH metre between the 3rd and the 4th rib 60 mm from the midline (EUTECH Instruments, Thermo Fisher Scientific). The pH metre had an automatic temperature compensator to adjust the pH for temperature. Before use, the pH metre and electrode were calibrated at pH 4 and pH 7 and was re-calibrated in pH buffers after every 4th reading. The carcasses were

then placed in a cold room, kept at an approximate temperature of 0 ºC for 24 h and then the cold carcass weights (CCW) were measured. The head from each carcass was then removed at the atlanto-occipital joint, the tail at the junction of the third and fourth sacral vertebrae and the flare fat, kidneys, kidney fat, glands and remaining parts of the diaphragm were also removed. Carcasses were then split into two parts along the median plane from the remaining sacral vertebra to the first cervical vertebra with a carcass splitting band saw. Carcass length (CL) was measured from the first rib to the pubic bone using a measuring tape. Backfat measurements were taken at first rib (DFT1), last rib (DFT2) and last lumbar vertebra (DFT3). All other carcass measurements were taken from the left side. A cut was made between the l0th and 11th ribs and carried on through the spinal column. The P2 fat measurement was taken on each carcass with vernier callipers over the eye muscle, 60 mm from the carcass midline. Eye muscle length (EML) and three measurements of the eye muscle width (EMW) were taken from the cut interface. Lean meat percentage (Lean %) was calculated using the formula of Bruwer (1992) presented below:

Lean % = 72.5114 – 0.4618V + 0.0547S

where V is the fat thickness (mm) and S is the muscle depth (mm).

The eye muscle area (EMA) was estimated using the formula proposed by Zhang *et al.* (2007) as:

EMA = EML x EML x 0.7

Where EMW was the average of the three width measurements of the eye muscle

From the same cut where P2 measurements were taken, a sample joint measuring 2.5 cm thick and 16 cm long measured along the surface of the back of the eye muscle was cut out and weighed. This sample joint was placed in a netlon bag and inserted in a small plastic bag which was then tied in such a way as to prevent the sample joint from touching the bottom of the plastic bag or air coming into the bag. They were then stored in a refrigerator between 0 and 5 ºC for 24 h after which the mass of the water lost was calculated from the weight of the water in the bag and used to calculate drip loss (DL). Thereafter, the primal cuts (shoulder, hindquarter and rib) from the carcasses were removed on a stationary band saw. The shoulder was removed by cutting between the third and fourth ribs caudally and the junction of the caudal edge of the second rib with the sternum cranially, with the front trotter removed by cutting through the metacarpal region (at the joint of the carpal bones and the radius and ulna) and weighed to get shoulder weight (SW). The rib was cut from between the fourth and twelfth thoracic vertebrae dorsally and along a parallel line 16 cm from the spinal cord midline ventrally. It was weighed to obtain the rib weight (RW). The hind leg was removed between the second and third sacral vertebrae perpendicular to the stretched leg and at the hock joint distally and weighed to get the hindquarter weight (HQW). It was also measured to get the hindquarter length (HQL), from the ischiopubic symphysis to the hock joint and the hindquarter circumference (HQC) in the area of maximum amplitude near the base of the tail. The RW, SW and HQW were then each presented as a proportion of CCW to give RWP, SWP and HQWP respectively.

7.2.4 Statistical analyses

Growth performance and carcass measurements were analysed according to a factorial arrangement of treatments with two breeds (SAWIP and LW x LR), and three diets (CON, LMC, HMC) separately for the GRO and FIN using the GLM procedure of the statistical package of SAS (SAS Inst. Inc., Cary, NC). All data were tested for normality and homogeneity and comparisons were made to the 95 % significance level and tendencies were considered at $0.05 < P \leq 0.10$. The P2 data were square-root transformed to achieve normality. Metabolic weight (Initial body $BW^{0.75}$) was used as a covariate in the analysis of ADFI. The following model was used for the analyses:

$$Y_{ijk} = \mu + D_i + B_j + (D*B)_{ij} + e_{ijk}$$

Where Y_{ijk} - growth, carcass and serum metabolite measurement for the diet observation (i), the breed (j) and age (k); μ - overall mean; D_i - ith effect of diet (CON, LMC, HMC); B_j - jth effect of breed (SAWIP, LW x LR); $(D*B)_{ijk,}$- interactions of diet and breed and e_{ijk} - random error. The Bonferroni method and the PDIFF statistic of SAS (SAS Inst. Inc., Cary, NC) were used to separate the means.

7.3 Results

7.3.1 Growth performance

Growth performance measurements of grower and finisher South African Windsnyer-type Indigenous (SAWIP) and Large White x Landrace (LW x LR) crossbred pigs fed diets containing low (LMC) and high (HMC) levels of ensiled maize cobs are shown in Table 7.1. There were no breed x diet interactions for final weight (FW), ADFI, DMI, ADG and gain:feed (G:F) ratio for the growers and finishers. The LW x LR crosses had greater ($P < 0.05$) FW, ADFI, DMI ADG and G:F ratios than the SAWIP at both the grower and finisher stages. The SAWIP consumed more feed per $BW^{0.75}$ than LW x LR crosses at grower stage while LW x LR crosses consumed more than SAWIP at finisher stage ($P < 0.05$). The LW x LR finishers' ADFI per $BW^{0.75}$ for the HMC diet was greater ($P < 0.05$) than for the CON diet. The finishers' G:F ratio was greater ($P < 0.05$) in the CON than in the HMC diet.

7.3.2 Carcass traits

Carcass traits of grower and finisher SAWIP and LW x LR crosses pigs are shown in Table 7.2. There were breed x diet interactions ($P < 0.05$) for P2 measurements and drip loss (DL) in the finishers. The SAWIP on HMC diet had greater ($P < 0.05$) P2 measurement than those on LMC diet while the LW x LR crosses' P2

measurements on similar diets were not different. Drip loss of LW x LR carcasses on the CON diet was greater (P < 0.05) than those on LMC diet but it was similar for SAWIP carcasses on CON and LMC diets. The LW x LR growers and finishers had greater (P < 0.05) WCW, CCW, CL, DL, pH_{24}, EMA and Lean % than those of SAWIP growers and finishers. The LW x LR finishers on the CON diet had greater (P < 0.05) WCW and CCW than those on the HMC and LMC diets.

Table 7.1 Growth performances of grower and finisher South African Windsnyer-type Indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) pigs fed diets containing ensiled maize cobs

		Parameter[1]					
	Diet[3]	FW, kg	ADFI, kg·	ADFI, kg·BW$^{0.75}$	DMI, kg	ADG, kg	G:F, kg·kg^{-1}
GROWERS							
	CON	29.9^{a}	1.4^{a}	0.13^{bc}	1.2^{a}	0.48^{a}	0.35^{ab}
SAWIP	LMC	29.2^{a}	1.4^{a}	0.14^{c}	1.3^{a}	0.47^{a}	0.33^{a}
	HMC	26.5^{a}	1.3^{a}	0.13^{c}	1.1^{a}	0.41^{a}	0.33^{a}
	CON	47.3^{b}	1.7^{b}	0.11^{a}	1.5^{b}	0.67^{b}	0.41^{b}
LW x LR	LMC	46.4^{b}	1.7^{b}	0.11^{ab}	1.3^{b}	0.66^{b}	0.39^{b}
	HMC	46.8^{b}	1.8^{b}	0.12^{ab}	1.6^{b}	0.64^{b}	0.36^{ab}
SD		4.12	0.17	0.013	0.16	0.07	0.05
FINISHERS							
	CON	60.1^{a}	1.3^{a}	0.09^{a}	1.2^{a}	0.54^{a}	0.41^{c}
SAWIP	LMC	58.8^{a}	1.5^{a}	0.10^{ab}	1.4^{a}	0.52^{a}	0.34^{ab}
	HMC	55.3^{a}	1.5^{a}	0.10^{ab}	1.4^{a}	0.48^{a}	0.32^{a}
	CON	89.4^{b}	2.5^{b}	0.12^{bc}	2.2^{b}	1.07^{b}	0.44^{c}
LW x LR	LMC	88.4^{b}	2.5^{b}	0.12^{c}	2.2^{b}	1.02^{b}	0.40^{c}
	HMC	82.7^{b}	2.6^{b}	0.14^{d}	2.4^{b}	0.96^{b}	0.37^{b}
SD		8.81	0.265	0.01	0.24	0.11	0.03

^{a,b} Within a column means with different superscripts differ (*P*<0.05)
Diet and diet*breed had no effects (P<0.05); Breed was significant (P<0.001)
[1]FW – Final weight; G:F – Gain to Feed ratio;
metabolic body weight (BW$^{0.75}$) was calculated as the mean of the Initial BW$^{0.75}$ and Final BW$^{0.75}$
[2]GROWERS SAWIP n=10 per diet; LW x LR n=18 each for LMC and MHC, n=14 for CON
FINISHERS SAWIP n=5 per diet; LW x LR, n= 9 each for LMC and HMC, n=7 for CON
[3]CON – Control diet; LMC - low maize cob inclusion diet; HMC – high maize cob inclusion diet

Primal pork cuts measurements in SAWIP and LW x LR pigs fed diets containing ensiled maize cobs are shown in Table 7.3. There were diet x breed interactions for dorsal fat thickness at first rib (DFT1), dorsal fat thickness at last lumbar vertebra (DFT3), backfat thickness (BFT) and hind quarter weight proportion (HQWP) in the growers. The DFT1, DFT3, BFT and HQWP of SAWIP growers on the CON and LMC diets were greater (P < 0.05) than those on the HMC diet and there were no differences in DFT1, DFT3, BFT and HQWP measurements on the CON, LMC and HMC diets. The dorsal fat thickness at last rib (DFT2) and

DFT3 measurements of SAWIP growers on CON and LMC diets were greater (P < 0.05) than those on the HMC diet. The LW x LR growers and finishers had greater values (P < 0.05) of HQL, HQC, HQWP and SWP than the SAWIP growers and finishers respectively. The SAWIP growers and finishers had greater values (P < 0.05) of DFT1, DFT2, DFT3 and BFT than the LW x LR growers and finishers respectively.

Table 7.2 Carcass traits of grower and finisher South African Windsnyer-type Indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) pigs fed diets containing ensiled maize cobs at low (LMC) and high (HMC) inclusion levels

		Parameter[1]								
Breed	Diet[3]	WCW, kg	CCW, kg	DP %	CL, cm	P2, mm	pH_{24}	DL, %	EMA, cm^2	Lean %
GROWERS										
	CON	22.3^{a}	21.6^{a}	74.2	50.0^{a}	21.0^{e}	5.2^{a}	1.7^{ab}	9.2^{a}	64.0^{a}
SAWIP	LMC	22.5^{a}	21.9^{a}	74.7	52.1^{a}	18.2^{bc}	5.5^{ab}	1.9^{ab}	10.4^{a}	65.5^{ab}
	HMC	20.0^{a}	19.4^{a}	75.4	49.7^{a}	16.2^{b}	5.6^{ab}	1.3^{a}	9.6^{a}	66.3^{b}
	CON	35.5^{b}	34.7^{b}	75.1	63.4^{b}	9.8^{a}	6.1^{b}	2.7^{b}	18.8^{b}	69.8^{c}
LW x LR	LMC	35.3^{b}	34.6^{b}	76.7	64.2^{b}	8.8^{a}	6.0^{b}	2.9^{b}	19.5^{b}	70.1^{c}
	HMC	35.4^{b}	34.8^{b}	75.7	64.3^{b}	7.8^{a}	5.9^{b}	2.0^{ab}	20.2^{b}	70.7^{c}
SD		3.49	3.40	2.72	2.23	2.55	0.38	0.89	3.00	1.20
Diet		0.607	0.630	0.762	0.317	0.036	0.827	0.123	0.805	0.032
Breed		<0.0001	<0.0001	0.305	<0.0001	<0.0001	0.001	0.016	<0.0001	<0.0001
Diet x Breed		0.552	0.542	0.707	0.322	0.534	0.512	0.919	0.800	0.451
FINISHERS										
	CON	44.8^{a}	44.0^{a}	74.6	64.0^{a}	25.5^{bc}	6.2^{b}	2.6^{ab}	18.0^{a}	61.4^{a}
SAWIP	LMC	46.0^{a}	45.2^{a}	72.7	65.2^{a}	20.9^{b}	5.4^{a}	2.2^{a}	16.5^{a}	63.5^{a}
	HMC	40.7^{a}	39.9^{a}	73.5	63.9^{a}	26.6^{c}	5.8^{ab}	2.2^{a}	15.6^{a}	61.2^{a}
	CON	72.2^{c}	70.8^{c}	72.6	78.2^{c}	12.3^{a}	5.8^{ab}	7.3^{c}	35.2^{b}	69.3^{b}
LW x LR	LMC	64.1^{b}	62.8^{b}	72.6	74.4^{b}	12.6^{a}	6.1^{b}	3.2^{ab}	31.5^{b}	69.4^{b}
	HMC	60.2^{b}	58.8^{b}	72.8	74.4^{b}	10.4^{a}	6.0^{b}	3.8^{b}	31.4^{b}	70.4^{b}
SD		4.97	4.96	2.55	3.10	4.26	0.52	1.22	4.34	2.22
Diet		0.004	0.004	0.702	0.458	0.415	0.481	0.004	0.363	0.557
Breed		<0.0001	<0.0001	0.337	<0.0001	<0.0001	0.284	<0.0001	<0.0001	<0.0001
Breed x Diet		0.205	0.219	0.771	0.281	0.035	0.102	0.024	0.873	0.101

[a,b] Within a column means with different superscripts differ (*P*<0.05)

[1]WCW- warm carcass weights; CCW- cold carcass weights; DP - dressing percentage; CL - carcass length; P2 - backfat thickness; pH_{24} – pH at 24 hours; EMA - eye muscle area; Lean % - lean percentage; DL - drip loss

[2]GROWERS SAWIP n=10 per diet; LW x LR n=18 each for LMC and MHC, n=14 for CON
FINISHERS SAWIP n=5 per diet; LW x LR, n= 9 each for LMC and HMC, n=7 for CON

[3]CON – Control diet; LMC - low maize cob inclusion diet; HMC – high maize cob inclusion diet
SAWIP n=5 per diet; LW x LR n=9 for LMC and HMC; n= 8 for CON

Table 7.3 Primal pork cuts measurements in South African Windsnyer-type Indigenous pigs (SAWIP) and Large White x Landrace (LW x LR) pigs fed diets containing ensiled maize cobs at low (LMC) and high (HMC) inclusion levels

		Parameter[1]								
Breed	Diet[3]	HQL, cm	HQC, cm	DFT1, mm	DFT2, mm	DFT3, mm	BFT, mm	HQWP %	RWP %	SWP %
GROWERS										
	CON	24.5^{ab}	34.1^{a}	30.0^{cd}	28.0^{e}	27.3^{c}	27.1^{e}	19.6^{bc}	9.4^{ab}	10.9^{a}
SAWIP	LMC	24.6^{ab}	38.7^{b}	34.0^{d}	21.8^{e}	26.6^{c}	26.3^{e}	18.1^{ab}	9.0^{a}	12.9^{b}
	HMC	23.8^{a}	36.3^{ab}	25.8^{bc}	17.0^{b}	19.6^{b}	20.8^{b}	17.8^{a}	10.8^{b}	12.0^{ab}
	CON	26.0^{bc}	52.0^{c}	20.3^{ab}	10.5^{a}	13.0^{a}	13.8^{a}	19.7^{c}	11.5^{b}	12.2^{ab}
LW x LR	LMC	26.6^{c}	52.2^{c}	18.9^{a}	10.1^{a}	11.7^{a}	13.6^{a}	20.9^{c}	10.7^{b}	12.5^{ab}
	HMC	26.5^{c}	51.9^{c}	19.9^{a}	9.0^{a}	12.6^{a}	13.8^{a}	20.2^{c}	10.9^{b}	12.4^{ab}
SD		1.07	2.08	4.32	3.82	3.51	3.26	0.76	1.02	0.99
Diet		0.509	0.065	0.124	0.019	0.039	0.073	0.151	0.109	0.164
Breed		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.009	0.400
Diet x Breed		0.523	0.124	0.041	0.114	0.013	0.050	0.038	0.140	0.372
FINISHERS										
	CON	30.0^{bc}	50.7^{a}	41.0^{c}	26.0^{b}	29.0^{b}	32.0^{b}	19.1^{ab}	11.6	10.5^{a}
SAWIP	LMC	28.2^{ab}	51.2^{a}	35.4^{c}	25.9^{b}	27.8^{b}	29.7^{b}	19.7^{ab}	11.8	11.1^{ab}
	HMC	26.3^{a}	50.6^{a}	37.9^{c}	27.4^{b}	29.3^{b}	31.5^{b}	18.7^{a}	12.2	10.6^{a}
	CON	31.2^{e}	64.3^{b}	30.8^{bc}	17.0^{a}	14.0^{a}	22.3^{a}	20.4^{b}	12.0	11.9^{b}
LW x LR	LMC	31.4^{e}	65.7^{b}	27.1^{ab}	16.9^{a}	17.8^{a}	20.6^{a}	20.2^{b}	10.9	11.3^{ab}
	HMC	31.3^{e}	63.2^{b}	22.4^{a}	14.0^{a}	17.0^{a}	17.8^{a}	20.5^{b}	11.2	11.6^{b}
SD		2.11	3.20	6.37	6.17	4.94	5.11	1.12	1.19	0.70
Diet		0.204	0.388	0.203	0.944	0.806	0.626	0.655	0.629	0.861
Breed		0.0003	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.009	0.283	0.003
Breed x Diet		0.181	0.706	0.268	0.546	0.582	0.403	0.269	0.517	0.139

[a,b] Means with different letters in a column differ significantly (P<0.05)
[1]HQL - hind quarter length; HQC - hind quarter circumference; DFT1 - dorsal fat thickness at first rib; DFT2 - dorsal fat thickness at last rib; DFT3 - dorsal fat thickness at last lumbar vertebra; BFT – backfat thickness; HQWP - hind quarter weight proportion; RWP - rib weight proportion; SWP- shoulder weight proportion
[2]GROWERS SAWIP n=10 per diet; LW x LR n=18 each for LMC and MHC, n=16 for CON
FINISHERS SAWIP n=5 per diet; LW x LR, n= 9 each for LMC and HMC, n=7 for CON
[3]CON – Control diet; LMC - low maize cob inclusion diet; HMC – high maize cob inclusion diet

7.4 Discussion

7.4.1 Growth performance

The SAWIP is a slow growing pig and has a smaller frame than the LW x LR, therefore the lower final weights and carcass weights were as expected. The SAWIP growers' ability to consume more feed per

metabolic weight than the LW x LR growers may however be a reflection of an adaptation to survive under marginal nutritional resources. The LW x LR growers would naturally consume more feed than SAWIP growers because of their bigger body size and gut capacity (Whittemore *et al*., 2003; Thacker & Haq, 2009). Frank *et al*. (1983), Ndindana *et al*. (2002) and Kanengoni *et al*. (2004) reported a reduction in average daily gain and feed intake as maize cob inclusion level increased. There were no similar reductions in this study. The differences between these studies and the current study could be attributed to ensiling although other factors such as the different experimental conditions and the differences in genotypes cannot be discounted.

7.4.2 Carcass traits

Although the LW x LR had greater carcass weights, the SAWIP had more uniform carcass weights at the finisher phases among the three diets. This could be interpreted as the SAWIP being more efficient than the LW x LR at utilizing the diets containing maize cobs. Alternatively it could be that the diets with higher maize cobs did not supply sufficient nutrients to meet the requirements of the LW x LR. Diets containing maize cobs did not have lower DP's as was expected. High fibre diets increase weights of visceral organs in pigs (Montagne *et al*., 2003). Stanogias & Pearce (1985) reported that prolonged intake of maize cob-supplemented diets by grower pigs led to a hypertrophy and increased weights of segments of the gastrointestinal tract, which was not observed in the current study.

The greater drip loss values in LW x LR than in the SAWIP are difficult to explain. Drip loss in pork is affected by numerous and complex factors including rate of pH decline and ultimate pH, the presence of the halothane gene and transportation among others (Pérez *et al*., 2002; Rosenvold & Andersen, 2003; Fischer, 2007). Although drip loss is of economic importance, the mechanism behind this phenomenon has not been extensively studied (Otto *et al*., 2007). In addition, because the LW x LR had a greater ultimate pH the DL results contradict the assertion by Huff-Longergan *et al*. (2002) that a higher ultimate pH is associated with better water holding capacity, translating into lower drip losses during storage.

Breed of pig influenced length of the carcass in the study more than diet did, with the exception of LW x LR finishers on the CON diet, which had longer carcasses than other pigs on the LMC and HMC diets. Carcass length affects the weights of the most valuable meat cuts (Poto *et al*., 2007) and determines the amount of rashers of back bacon obtained (Kanengoni *et al*., 2004). English *et al*. (1988) stated carcass weight and the genotype of the pig largely influence CL. This implies that the diets did not influence carcass weights sufficiently as to affect the carcass length in either genotype except for the LW x LR finishers on the CON diet. Given that the LW x LR crosses are improved genetically for body conformation, unlike the SAWIP, which have short carcasses, this was expected. Unimproved genotypes like the SAWIP also have a smaller eye muscle compared to improved genotypes. Generally, the SAWIP had more subcutaneous fat than the LW x LR and this showed in a lower lean percentage. The lean percentage values for the SAWIP has to be interpreted cautiously because the equations used were developed using improved breeds. The fat and lean measurements were not affected by the diet as much as they were by breed. Backfat thickness measures in the SAWIP growers were similar to the SAWIP finishers' unlike in the LW x LR where the differences

between the growers' and finishers' were greater. This suggests that the laying on of backfat in the SAWIP starts quite early. This corroborates the observation by Mersmann (1991) that pigs selected for obesity reach compositional maturity at a younger age compared with lean pigs. Inclusion of ensiled maize cobs in diets however did not negatively affect some measurements of selected important commercial pork cuts in South Africa comprising the hindquarter, ribs and the shoulder. The hindquarter length, circumference and weight proportion and the shoulder weight proportion measures were affected mainly by breed similar to the CL.

7.5 Conclusions

The breed of pig influenced most of the growth performance and carcass parameters more than the diet did. The SAWIP demonstrated an adaptation to high fibre diets by consuming more feed than the LW x LR per metabolic body weight at the grower stage. The SAWIP however put on backfat early unlike the LW x LR and the mechanisms governing this tendency need to be explored further. Since the inclusion of ensiled maize cobs in diets did not affect negatively the selected important commercial pork cuts in South Africa, this could imply that they have a greater role as a pig feed resource.

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Chapter 8

8 General discussion and conclusion

8.1 General discussion

Competition with humans and the biofuel industry for feed resources is constraining the pig industry, which already has a narrow range of feed ingredients (Smale *et al*., 2013). Yet there are underutilized resources such as maize cobs, which have the potential to mitigate against ingredient shortage (Ndindana *et al*., 2002; Kanengoni *et al*., 2004). This study proposed that maize cobs be regarded as a valuable pig feed ingredient and innovative ways be applied to increase their utilisation. It was proposed that they should be processed through ensiling with exogenous enzymes to reduce the fibre levels before embarking on feeding trials. This study used maize cobs and two divergent breeds, the SAWIP and the LW x LR in the model.

The hypothesis tested in Chapter 3 was that ensiling of maize cobs reduces fibre levels. Four combinations of additives were compared to determine fermentation, aerobic stability, and nutrient composition.
The five treatments were: (i) control (maize cobs without additives; CON),
(ii) maize cobs with sugarcane molasses only (MOL),
(iii) maize cobs with a combination of sugarcane molasses and whey (MOW),
(iv) maize cobs with a combination of sugarcane molasses, whey and exogenous enzyme at 0.5 g/kg maize cob mixture (ENZ1) and,
(v) maize cobs with a combination of sugarcane molasses, whey and exogenous enzyme at 1 g/kg maize cob mixture (ENZ2).
They were ensiled in 1.5 L anaerobic glass jars over 32 days and assessed for fermentability and nutrient composition. Ensiling maize cobs with molasses, whey and exogenous enzymes did not improve fermentation characteristics but exogenous enzymes reduced fibre fractions and energy content of maize cob silages. The hypothesis was accepted and maize cobs were ensiled without additive for diet formulations and feeding trials.

Chapter 4 tested the hypothesis that diets containing ensiled maize cobs improve feed preference, nutrient digestibility and fermentability in the colon when fed to LW x LR and SAWIP pigs. Three treatments; CON (control diet without maize cobs), LMC (low maize cob level diet containing 100 g maize cobs/kg) and HMC (high maize cob level diet containing 200g maize cobs/kg) were assessed. Preference was assessed in 64 LW x LR and 30 SAWIP by offering the pigs two choices between a common reference diet and a test diet. Apparent total tract digestibility (ATTD) and colon fermentation were determined using 15 LW x LR and 15 SAWIP pigs. The SAWIP preferred the CON diet compared to diets with maize cobs while LW x LR had no preference. The SAWIP had higher digestibility coefficients of DM, OM, CP, GE, ADF and NDF, than the LW x LR. The HMC diet had higher digestibility values of CP, EE, ADF, hemicellulose and NDF than the CON

diet. The LW x LR had higher colonic concentrations of total volatile fatty acids (VFA) than the SAWIP implying better fermentation. The CON and LMC diets resulted in more VFA production than the HMC diet. The hypothesis was rejected that ensiled maize cobs in diets improve preference and fermentability in the colon. However the hypothesis that maize cobs improved total tract digestibility was accepted.

The fifth Chapter tested the hypothesis that indigenous pig breeds have a myriad of structural carbohydrate digesting colon microbes, which enhance fibre degradation. The study compared the composition of faecal bacterial communities in SAWIP and LW x LR crosses using metagenomic pyrosequencing of 16S rRNA genes in an effort to explain the differences in fibre utilization between these breeds. Eight LW x LR and five SAWIP pigs were evaluated on a diet without maize cobs (CON) and a diet containing 20 % ensiled maize cobs (HMC) in a completely randomized block design after 8 weeks on the diets. Analysis of the microbiome revealed differences occurring between the two breeds but not between the diets. The hypothesis was accepted.

Chapter 6 tested the hypothesis that specific proteins act as biomarkers that identify pigs with an enhanced ability to digest and utilise high fibre diets. The study compared serum metabolite and liver histometry responses in indigenous pigs and commercial pigs fed diets containing ensiled maize cobs. The study also established proof of principle on the use of a sodium dodecyl sulphate polyacrylamide gel electrophoresis/Matrix-assisted laser desorption ionization mass spectrometry (SDS-PAGE/MALDI MS) workflow to evaluate differences in serum and liver protein profiles of pigs fed high fibre diets. Twenty-four LW x LR and 15 SAWIP were assessed in the study. They were fed a control diet (CON), a low ensiled maize cob inclusion (LMC) and a high ensiled maize cob inclusion (HMC) diet in a completely randomized block design. The study demonstrated differences in serum and liver proteins and in serum metabolite levels related to diet and breed. The hypothesis was accepted.

Chapter 7 tested the hypothesis that diets containing maize cobs improved growth performance and carcass measurements in LW x LR and SAWIP pigs. Fifty Large White × Landrace crossbred pigs (LW x LR) and 30 South African Windsnyer-type Indigenous pigs (SAWIP) were assessed. They were fed a control diet (CON), a low ensiled maize cob inclusion (LMC) and a high-ensiled maize cob inclusion (HMC) diet in a completely randomized block design. Response to the diets was breed dependent. For example, daily gain in the SAWIP was similar in all the diets at the grower and finisher stages but was higher for LW x LR pigs on the CON diet than the LMC and HMC diets in the finisher stage. Ensiled maize cob diets did not affect negatively selected important commercial pork cuts. The hypothesis was rejected since there was no improvement in growth performance and carcass measurements in both breeds. It was noted however that there were no negative growth and carcass measures in the SAWIP.

8.2 Recommendations

Classical nutritional, biochemical and histological approaches have failed to elucidate the extent the pigs' ability to digest and utilise fibrous diets is influenced by breed, age of the animal or the characteristics of the fibre (Hedemann *et al*., 2006; Morel *et al*., 2006; Degen *et al*., 2007; von Heimendahl *et al*., 2010). A dearth of knowledge in the underlying mechanisms involved in fibre utilisation limits strategies to increase the use of the fibrous ingredients. Therefore, each dietary source has to be evaluated fully to elucidate its merits. Unfortunately, owing to limitations in terms of resources only a few pigs were assessed. There were too few indigenous pigs. This may have compromised the quality of the data especially as relating to microbiome and proteomic profiling. Nonetheless the results obtained show that pigs have an inherent capacity to utilize fibre.

It is recommended that:

1. Further work should investigate the impact of ensiling maize cobs with only exogenous enzymes and for dose response studies using different concentrations of enzymes to determine the optimum concentration.
2. Analysis of the microbiome revealed differences due to breed but not diet. The results suggested some faecal bacterial communities were largely similar between LW x LR and SAWIP pigs fed a control diet and a diet containing ensiled maize cobs, though the sampling size may have been a limiting factor to observing differences. There is need to define further the relationship between the faecal microbiome and pigs' efficiency in digesting fibrous diets and this will allow for additional gains in performance and productivity. If some of these bacteria are identified and classified on their function, they can be evaluated as potential probiotics.
3. A significant proportion of OTU's were not assigned any family or genus and these will need to be investigated further in future studies
4. Further work to identify the proteins from the protein bands using higher resolution large format 2D SDS-PAGE and with more sensitive non-gel based methods is necessary to validate the preliminary findings of this particular work. In addition, Nano LC-MS/MS (Liquid chromatography mass spectrometry) can characterize the tryptic digests of the purified protein band on SDS-PAGE and determine their function.
5. Through biochemical profiling, the study showed that protein levels provided in the diet were higher than SAWIP's requirements. This should lead to further studies to determine the actual protein requirements of SAWIP.

8.3 References

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